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Published in London, United Kingdom







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http://dx.doi.org/10.5772/intechopen.73916
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First published in London, United Kingdom, 2020 by IntechOpen
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, 7th floor, 10 Lower Thames Street, London, EC3R 6AF, United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

Cell Growth
Edited by Biba Vikas and Michael Fasullo
p. cm.
Print ISBN 978-1-78923-806-8
Online ISBN 978-1-78984-557-0
eBook (PDF) ISBN 978-1-78985-311-7

Meet the editors

Dr. Biba Vikas carried out her post-doctoral studies on cancer biology from KSCSTE, Jawharlal Nehru Tropical Botanic Garden and Research Institute at Thiruvannathapuram, India. She completed her PhD in cancer biology – biotechnology at the Regional Cancer Centre, Thiruvanthapuram, India. She worked as a post-doctoral research associate in CSIR- National Institute for Interdisciplinary Science and Technology, Trivandrum, India. She started working as a research fellow at the Regional Cancer Centre, Thiruvananthapuram, India. Her areas of expertise are cancer research, microbiology, molecular biology, tissue culture, drug delivery, cell-based assays, and phytochemical analysis.

Michael Fasullo, Ph.D., earned his BSc degree from the Massachusetts Institute of Technology, his Ph.D. from Stanford University, and completed his postdoctoral studies with Dr. Rodney Rothstein at Columbia University. His research focuses on understanding the genotoxicity of DNA damaging agents using budding yeast as a model system. He developed assays for quantifying DNA damage-associated recombination. Initially his studies focused on how cell cycle checkpoints suppress radiation-induced rearrangements. By introducing human cytochrome P450 genes into yeast strains, he now focuses on genome profiling of carcinogen resistance to mycotoxins and heterocyclic aromatic amines. He has published over thirty publications in this field and his research has been supported by National Institutes of Health, the Department of Defense, and the March of Dimes.

Contents

Preface

This book on cell growth is the ideal resource for scientists who want to know more about cell growth. This important new text provides information on the following topics: cell growth with plant growth hormones, kinetic studies on cell growth, growth of fungal cells and the production of mycotoxins, and cell growth measurement. The term *cell growth* can be defined as biological cell development and cell division or reproduction. Cell growth is referred to as proliferation of a cell (mother cell) as it grows and divides to produce two daughter cells (M phase). Cell proliferation requires nutrients, energy, and biosynthetic activity to duplicate all macromolecular components during each passage through the cell cycle. Cell growth is an important feature of cell cycle entry and proliferation of the cell cycle. In the cell cycle, cells replicate themselves in an organized manner. It is the stage in which cells are preparing for the next division, biochemical activities, and reactions.

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Chapter 1

Plant Growth Hormones

Amira Shawky Soliman

Abstract

Many factors can cause and affect cell growth in the plant such as external (environmental) and internal factors; one of the most important internal factors is plant growth hormones. Many hormones required for cell growth, such as auxins, gibberellins, brassinosteroids, ethylene, jasmonates, salicylic acid, strigolactones and cytokinins which able to accelerate or promote growth, but, some hormone-like abscisic acid has an adverse effect on growth which increases seed dormancy by inhibiting cell growth. Also, plant hormones are able to breakdowns dormancy for many plants and can alleviate abiotic stress (salinity, extreme temperatures and, drought,...) which led to enhance germination and improve growth for many plants, whether naturally occurring in the plant or by adding it to the plant in its artificially formed or in the form of bio- or nano-fertilization in order to increase the productivity and improve its efficiency under extreme conditions. Therefore, this chapter will highlight and will provide data for the positive or/and negative effect of these hormones on many plants to achieve a rapid germination method. It will also shed light on the relationship of these hormones to some enzymes to accelerate growth.

Keywords: plant hormones, seed germination, dormancy, cell growth, inhibition

1. Introduction

Plant hormones (phytohormones) are not nutrients, but chemicals and not all plant cells respond to hormones, but those cells that do are programmed to respond at specific points in their growth cycle. The greatest effects occur at specific stages during the cell's life, with diminished effects occurring before or after this period [1].

Plants need hormones at very specific times during plant growth and at specific locations. They also need to disengage the effects that hormones have when they are no longer needed. The production of hormones occurs very often at sites of active growth within the meristems, before the cells have fully differentiated. After production, they have sometimes moved to other parts of the plant, where they cause an immediate effect; or they can be stored in cells to be released later. Plants can also break down hormones chemically, effectively destroying them. Plant hormones frequently regulate the concentrations of other plant hormones [2, 3].

2. Importance of plant hormones

The small amounts of plant hormones promote, control, influence and develop the growth from embryo to reproductive development, also, stress tolerance and pathogen defense. According to the importance of plant hormones in this chapter will be divided into two main points: first: the effect of plant hormones on

germination and growth of plants under internal or external suitable conditions, second: the effect of plant hormones on the germination and growth of plants under internal or external unsuitable environmental conditions.

2.1 The effect of plant hormones on germination and growth of plants under internal or external suitable conditions

Seed germination is attracted to the effective growth of the embryo when appropriate environmental conditions are present, leading to seed rupture and the appearance of a small plant. There are five basic steps to germination: water imbibition, enzyme activation, initiation of embryo growth, rupture of the seed coat and emergence of seedling, seedling establishment [4, 5].

In the second step stage of germination (enzyme activation), after the absorption of water through the natural openings in the casing of the seed and spread through the tissues of the seed, gibberellins which activate the formation of the hydrolytic enzymes, mainly α- amylase in the aleurone cells, which are responsible for hydrolysis of storage macro-molecules such as starch and proteins and convert them into available forms to the embryo, usage to increase in size, and raise the osmotic content of the seed, to increase water potential [6, 7].

In addition, plant hormones have an important role in plant growth not only germination such as cytokinins (CKs) which influence cell division, the formation of shoot and helping in delay tissues senescence [8, 9]. Also, the ratios of Cytokinins and auxins affect most major growth periods during a plant's lifetime [10]. Also, Peptide hormones, control of cell division, expansion, and play crucial roles in plant growth and development [11]. Furthermore, gibberellins (Gas) strongly promote cell elongation in seedlings [12, 13]. It can also affect cell cycling in plant [14]. Meanwhile, the responses of **n**itric oxide (NO) are in germination, cell death [15] and regulate plant cell organelle functions (e.g. mitochondria and ATP synthesis in chloroplasts) [16].

For enhances and increases plant hormones production in the plants, many studies have proved the need to add plant hormones either directly (GA3, kinetin and cytokinins) [17, 18] or indirectly (humic substances, manures, magnetite, natural zeolites, *Moringa* extract and bio-fertilization) to increase or accelerate the productivity of plant hormones in the plant [19] indicated that, the presence of organic matter represented in compost which a source of hormones like substances as auxin-like activity and gibberellin-like activity. Similar results were obtained from [20]. Ref. [21] concluded that it is also possible that the production of plant hormones influences symbiotic bacteria, such as nodule N_2 fixing bacteria. During the establishment of the soybean (*Glycine max* L.) and *Bradyrhizobium japonicum* N_2-fixing symbiosis, the production of plant hormones can determine the bacterial population in the nodules by, for example affecting the available substrate for the use of rhizobium. The other significant and interesting view of the effects of soil bacteria on the production of plant hormones is the alteration they may reason in plant signaling pathways, resulting in the output of plant hormones from the host plant [22, 23]. Ref. [24] concluded that the magnetic treatments have the same affected for phytohormone production. Ref. [25] reported that the highest mean values of IAA, GA, and CK (12.70, 13.71, 11.06 μg/g FW), respectively were recorded with compost and zeolite mixture in comparison with control. Ref. [26] concluded that the addition of a mixture of organic fertilizers and soil amendments led to significant increment in indigenous hormones characterized in **i**ndole **a**cetic **a**cid (IAA), gibberellic acid (GA3), and cytokinins (CK), which led to a significant increase in morphological growth, floral characteristics and chemical composition of *Oenothera biennis*. In contrast [27] found that the application of HA inhibits

indoleacetic acid (IAA) oxidase, thereby hindering the destruction of this plant growth hormone.

2.2 The effect of plant hormones on the germination and growth of plants under internal or external unsuitable environmental conditions

Sometimes even under favorable germination conditions (an adequate water supply, a suitable temperature and the normal composition of the atmosphere) seeds do not germinate. In this case, seeds are considered dormant. Seed dormancy is defined as an inactive phase in which the growth and development are deferred and the respiration is greatly reduced [28, 29]. Seed coat dormancy involves the mechanical restriction of the seed coat. GA releases this dormancy by increasing the embryo growth potential, and/or weakening the seed coat so the radical of the seedling can break through the seed coat. ABA affects the testa or seed coat growth characteristics, including thickness, and affects the GA-mediated embryo growth potential [5].

Hormones also can mediate endosperm dormancy: Endosperm in most seeds is composed of living tissue that can actively respond to hormones generated by the embryo. The endosperm often acts as a barrier to seed germination, playing a part in seed coat dormancy or in the germination process. Living cells respond to and also affect the ABA:GA ratio, and mediate cellular sensitivity; GA thus increases the embryo growth potential and can promote endosperm weakening. GA also affects both ABA-independent and ABA-inhibiting process within the endosperm [30]. In addition, [33] concluded that the prevented germination of some seeds of tomato [31], iris [32], and some varieties of cabbage was due to the present of inhibitors (ABA, parasorbic acid, and coumarin) which cases distributed in plants and to possess the property of inhibiting seed germination and other growth phenomena [5, 34].

Plant hormones affect seed germination and dormancy by acting on different parts of the seed such as [35] found that the inhibitors in seeds of peach were at least one of the factors controlling in germination by preventing or retarding cell division of the radical. In *Lupinus angustifolius*, the contents of auxins increased through the 5th day of germination and started to decrease on the 7th day. Oppositely, gibberellins contents were decreased first then increased later, so it was clear that there was inversely related between auxins and gibberellins [36]. The germination percentage and germination rate of four studied Acacias (*A. saligna*, *A. sophorae*, *A. cyclopis,* and *A. melanoxylon*) were correlated positively with endogenous promoting and negative with endogenous inhibiting substances in their cotyledons plus embryo [37].

The promotion of germination by gibberellin and cytokinins has been demonstrated in many seed species [38, 39]. Ref. [40] treated the seeds of *Acacia longifolia*, with GA3 at 100 and 200 ppm and found that the higher GA3 concentration (200 ppm) was more effective in increasing germination while the concentration of 500 ppm was the best in the case of *Acacia catechu* [41].

Ref. [42] found that fresh seed of *Acacia nilotica* and *Acacia albida* were fully germinated when soaked in a solution of GA_3 at 200 ppm for 12 h. While soaking seeds of *Acacia nilotica* in gibberellic acid (100 or 300 ppm for 16 h) was the best [43]. Ref. [44] studied the effect of GA3 at a concentration of (50 ppm) on 16 species (four Acacia species), and found a high germination percentage for all species.

The effect of gibberellic acid and cytokinins were also recognized on the germination of other plant species seeds. Ref. [45] studied the effect of Kinetin at different concentrations on the seed germination of *Acer tataricum*, and found the highest germination percentage at the concentration of 500 ppm. Ref. [46] found the best germination percentage on soaking the seeds of *Trifolium pratense* in 50 ppm 6-benzylaminopurine (6-BAP). Ref. [47] studied the effect of Kinetin at different conc. (10, 25, 50 and 100 ppm) in the seeds of *Cassia sophera*, and found the highest

germination percentage at 100 ppm. The treatment of freshly harvested and 1 year old seeds of soybean (*Glycine max*) with, 1 ppm 6-BAP increased the germination percentage from 50 to 85% in freshly harvested seeds and to 75% in the older seeds [48]. The effect of kinetin and 6-BAP on the seed germination of *Vicia faba* were studied, [49] found an increase in its germination percentage at the concentration of 100 ppm kinetin. While [50] found that, the highest germination percentage for faba bean (*Vicia faba* L.) was achieved at the concentration of 100 ppm 6-BAP. Also [37, 51] reported that the storage has an adverse effect on the hormone within the seeds of *Acacia saligna*, *Acacia Cyclopes*, *Acacia nilotica* and *Acacia albida*, which contained the lowest value of GA3, IAA and the highest content of phenols.

Plant hormones can also alleviate abiotic stress such as drought, extreme temperatures, and salinity [52, 53]. The action of these hormones in response to situations of stress can be developed through synergistic or antagonistic activities [54]. Also, [55] concluded that the plant growth regulators like ABA, JA, and ethylene are involved in the regulation of the plant response to abiotic stress. Cytokinins are also able to enhance seed germination by the alleviation of stresses such as salinity, drought, heavy metals and oxidative stress [56–59]. Ref. [60] found that GA_3 plays an important role in the growth and metabolism of microalgae *Chlorella vulgaris* exposed to heavy metal stress and its adaptation ability to a low-level polluted aquatic environment. Meanwhile, gibberellin leads to enhancement for *Zea mays* seedling growth and establishment under saline soil conditions by improving nutrient levels and membrane permeability [61]. Also, hormonal interactions between plant and rhizosphere bacteria can affect plant tolerance to stress. As such, the plant and bacteria can be genetically modified so that they can perform more optimally under a range of conditions, including stress [62].

The decreased cytokinin and gibberellic acid (GA3) and increased abscisic acid contents are often observed responding in plants subjected to environmental stresses [63, 64]. Exogenous application of plant growth regulators [such as cytokinin or antioxidants (ascorbic acid) [65], Moringa (*Moringa oleifera*) leaves extract [66, 67], humic acid (HA) [68], or seaweed extract (SE) [69] could be an alternative strategy to ameliorate, minimizing or alleviating the adverse effects of abiotic stress factors on plant growth which led to promoting plant growth and development metabolism in plants. Several studies also indicated that results on wheat [70]; and on spinach [71]. Ref. [72] reported that the foliar application of Moringa (*Moringa oleifera*) leaves extract MLE is proved to be the most effective PGR in reducing plant (*Lagerstroemia indica* L. seedlings) exposure to salinity stress.

Also, bio-fertilization has beneficial microorganisms that increasing plant hormones, which led to enhances yield, plant growth and nutrient uptake under various environmental conditions such as salinity [73–76], drought and low fertility supply [77–79], especially that some endomycorrhizal fungi (Arbuscular mycorrhizal fungi) have been proven to improve drought stress; they colonize bio-trophically the root cortex and develop an extra-metrical mycelium that helps the plants to acquire mineral nutrients from the soil particularly those, which are immobile. They can under drought conditions stimulate growth-regulating substances, increase photosynthesis, improve osmotic adjustment, optimize hormonal balance and enhance water uptake [80].

Numerous studies have found also, that it can be alleviation of salt stress on peanut [81]; on pumpkin plants [82]; on *Moringa peregrina* plants [83] by using foliar application of nano-fertilizers. Also, [84] reported that nano Zn-Fe oxide plays a significant role importance in alleviating salt stress, oxidative damages on plant cells by activation of certain antioxidant enzymes. In addition, [85] reported that the application of nano-oxide and bio-fertilizer reduced the negative effects of salinity due to its contributed to produce hormones.

3. Conclusions

This chapter was indicated by many studies that the plant hormones, including IAA, cytokinins, ethylene, gibberellins, and brassinosteroids, can positively affect seed germination and seedling growth, for many plants as mentioned previously in the chapter, under favorable conditions. While ABA has an adverse on affect seed germination and the growth.

Also, this chapter sheds the light on the important role of soil bacteria in the production of plant hormones or as an alternative in the case of the low rate of plant hormones in the plant, which led to hence seed germination, growth, and hence crop production.

In addition, this chapter provided many studies that prove that the plant hormones very important to overcome dormancy or growth under stress condition. Also, shed the lights on the importance of the exogenous application of plant growth regulators (cytokinin or antioxidants, Moringa leaves extract, humic acid, or seaweed extract, bio- or nano fertilizers) for enhancing the productivity of plant hormones which led to increased cell growth.

Finally, it can be stated that the plant hormones are essential for cell growth, whether under normal conditions or under stress conditions.

Conflict of interest

The author declares that she does not have any conflict of interest.

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References

[1] Swarup R, Perry P, Hagenbeek D, Van Der Straeten D, Beemster GT, Sandberg G, et al. Ethylene upregulates auxin biosynthesis in Arabidopsis seedlings to enhance inhibition of root cell elongation. The Plant Cell. 2007;**19**(7):2186-2196. DOI: 10.1105/tpc.107.052100

[2] Campbell N, Reec JB. Biology. 6th ed. San Francisco: Benjamin Cummings; 2002. ISBN: 978-0805366242

[3] Davies PJ. The plant hormones: Their nature, occurrence, and functions. Plant Hormones: Physiology, Biochemistry and Molecular Biology. 2010:1-15. DOI: 10.1007/978-1-4020-2686-7_1

[4] Copeland LO. Principles of Seed Science and Technology. USA: Burgess Publishing Company; 1976. pp. 55-58

[5] Meyer BS, Anderson DB. Plant Physiology. 2nd ed. Canada: D. Van Nostrand Company, Ltd; 1952. pp. 709-715

[6] Ikuma I, Thimann KV. Metabolic control of germination. In: The Physiology and Biochemistry of Seed Dormancy and Germination. Amsterdam, New York, Oxford: North Holland Publ. Co.; 1963

[7] Öpik H, Rolfe SA, Willis AJ, Street HE. The Physiology of Flowering Plants. 4th ed. Cambridge University Press; 2005. p. 191. ISBN 978-0-521-66251-2

[8] To JP, Kieber JJ. Cytokinin signaling: Two-components and more. Trends in Plant Science. 2008;**13**:85-92

[9] Santner A, Calderon-Villalobos L, Estelle M. Plant hormones are versatile chemical regulators of plant growth. Nature Chemical Biology. 2009;**5**:301-307

[10] Sipes DL, Einset JW. Cytokinin stimulation of abscission in lemon pistil explants. Journal of Plant Growth Regulation. 1983;**2**(1-3):73-80. DOI: 10.1007/BF02042235

[11] Lindsey K, Casson S, Chilley P. Peptides: New signalling molecules in plants. Trends in Plant Science. 2002;7(2):78-83. DOI: 10.1016/S0960-9822(01)00435-3

[12] Tsai FY, Lin CC, Kao CH. A comparative study of the effects of abscisic acid and methyl jasmonate on seedling growth of rice. Plant Growth Regulation. 1997;**21**(1):37-42. DOI: 10.1023/A:1005761804191

[13] Van der Knaap E, Kim JH, Kende H. A novel gibberellins induced gene from rice and its potential regulatory role in stem growth. Plant Physiology. 2000;**122**:695-704

[14] Hauvermale AL, Ariizumi T, Steber C. Gibberellin signaling: A theme and variations on DELLA repression. Plant Physiology. 2012;**160**:83-92

[15] Shapiro AD. Nitric oxide signaling in plants. Vitamins and Hormones. 2005;**72**:339-398. DOI: 10.1016/S0083-6729(05)72010-0

[16] Roszer T. Nitric oxide synthesis in the chloroplast. In: Roszer T, editor. The Biology of Subcellular Nitric Oxide. New York, London, Heidelberg, Springer; 2012. ISBN 978-94-007-2818-9

[17] Afroz A, Chaudhry Z, Khan R, Rashid H, Khan SA. Effect of GA3 on regeneration response of three tomato cultivars (*Lycopersicon esculentum*). Pakistan Journal of Botany. 2009;**41**(1):143-151

[18] Hyunggook K, Donggeun C, Inkyu K. Effect of growth regulator treatments on quality and growth in 'Gailiangmeru' grape (*Vitis* spp.). Acta Horticulturae. 2008;**772**:319-322

[19] Marek K, Skorupska A. Production of B-group vitamins by plant growth promoting *Pseudomonas fluorescens* strain 267 and importance of vitamins in the colonization and nodulation of red clover. Biology and Fertility of Soils. 2001;**33**:146-151

[20] Khalil FA, EL-Aref KAO. Bio-fertilizers as partner with mineral N-fertilizer for fertilizing wheat crop cultivar Sids 6. Journal of Agricultural Science, Mansoura University. 2001;**26**:8287-8295

[21] Ikeda S, Okubo T, Anda M, Nakashita H, Yasuda M, Sato S, et al. Community- and genome-based views of plant-associated bacteria: Plant–bacterial interactions in soybean and rice. Plant & Cell Physiology. 2010;**51**:1398-1410

[22] Cui H, Wang Y, Xue L, Chu J, Yan C, Fu J, et al. *Pseudomonas syringae* effector protein AvrB perturbs *Arabidopsis* hormone signaling by activating MAP kinase. Cell Host & Microbe. 2010;**7**:164-175

[23] Miransari M, Abrishamchi A, Khoshbakht K, Niknam V. Plant hormones as signals in arbuscular mycorrhizal symbiosis. Critical Reviews in Biotechnology. 2014;**34**(2):123-133. DOI: 10.3109/07388551

[24] Maheshwari LB. Magnetic treatment of irrigation water: Evaluation of its effects on vegetable crop yield and water productivity [thesis]. New South Wales: Western Sydney University; 2009

[25] Soliman AS, Abdelwahab MM. Response of *Adansonia digitata* to compost and zeolite in replacement of chemical fertilization. American-Eurasian Journal Of Agricultural & Environmental Sciences. 2013;**13**(2):198-206

[26] Abdelwahab MM, Soliman AS. Comparative study on the influence of organic fertilizer and soil amendments on evening primrose (*Oenothera biennis* L.). International Journal of Agricultural Research. 2017;**12**:52-63

[27] Mato MC, Olmedo MG, Mendez J. Inhibition of indoleacetic acid-oxidase by soil humic acids fractionated on sephadex. Soil Biology and Biochemistry. 1972;**4**:469-473

[28] Bewley and Black. Seeds Physiology of Development and Germination. 2nd ed. New York, USA: Plenum Press; 1994

[29] Crawley MJ. Plant Ecology. 2nd ed. UK: Blackwell Science Ltd; 1997

[30] Gerhard W. Seed Dormancy. In: The Seed Biology Place. Royal Holloway University of London; 2000

[31] Evenari M. Germination inhibitors. The Botanical Review. 1949;**15**:153-194

[32] Randolph LF, Cox LG. Factors influencing the germination of Iris seed and the relation of inhibiting substances to embryo dormancy. Proceedings of the American Society for Horticultural Science. 1943;**43**:284

[33] Cox Herald R, Clancy CF, Wolfe DM, Vanderscheer J. Report of Preliminary Studies on Tsutsugamushi (*Scrub typhus*). submitted to Natl. Research Council; 1945

[34] Veldstra H, Havinga E. Enzymologia. Amsterdam. 1945;**11**:373

[35] El-Khodary SES, Moustafa SM. Effect of a potent germination inhibitor of dormant Peach seeds on division of root cells. Proc. Soudi Bio. Soci. 1979;**3**:149-166

[36] Denisova GM, Dmitrieve TA, Viktorova VE. On the kinetics of endogenous growth substances in organs of seedlings of *Lupinus angustifolius*. In: Fiziologiya Rostovykh

Protsessov. Moscow: USSR; 1980. pp. 41-48

[37] Youssef EMA, Badawy ESM, H eikal EA, Sakr SS. Studies on the germination of different *Acacia* species. Bulletin of Faculty of Pharmacy, Cairo University. 1991;**42**(3):849-868

[38] Mayer AM, Poljakoff-Mayber A. The Germination of Seeds. 3rd ed. Oxford, UK: Pergamon Press; 1982

[39] Briggs DE. In: Milbrorrow BV, editor. Biosynthesis and Its Control in Plants. New York: Academics Press; 1973. p. 219

[40] AL-Kinany P. Effect of some pre-treatment on seed germination and subsequent development at *Acacia longifolia* seedling. Pakistan Journal of Forestry. 1981;**31**(3):81-88

[41] Singh SS, Sharma BN, Paliwal GS. Effect of Niagara and GA3 on seed germination, early seedling growth, and cotyledonary expansion of *Acacia catechu* Willd. Indian Journal of Forestry. 1985;**8**(1):41-46

[42] Krishnasamy V, Palaniappan M. Studies on seed dormancy in brinjal var. 'Annamalai'. South Indian horticulture. 1994;**38**(1):42-44

[43] Patel VS, Kukadia MU, Vashi BG, Jadeja DB. The improvement of germination and seedling growth of *Acacia nilotica* (Linn.) Wild. by scarification methods. Gujarat Agricultural Universities Research Journal. 1999;**21**(2):149-151

[44] Bell DT, King LA, Plummer JA. Ecophysiological effect of light quality and nitrate on seed germination in species from Western Australia. Australian Journal of Ecology. 2003;**24**(1):2-10

[45] Pozdova LM. Changes of cytokinin activity during breaking dormancy in seeds. Soviet Plant Physiology. 1985;**32**(2):368-374

[46] Kovalev VM, Shipova EV. Increasing the adaptive capacity of plants using physiologically active substances. In: Vestinik Sel'skohozyaistvennoi Nauki. Vol. 2. Moscow: USSR; 1987. pp. 74-78

[47] Chhavi T, Thakur C. Influence of different growth regulators on seed germination and seedling growth of *Cassia sophera* L. Acta Botanica Indica. 1989;**17**(2):187-191

[48] Fujiki T, Naito T, Akihama T. Promotion of germination and reduction of decrease of t RNA content in the embryo of aged seeds of soybean (*Glycine max* M.) treated with 6-benzylaminopurine. Japanese Journal of Breeding. 1989;**39**(1):1-7

[49] Gupta M. Effect of growth regulators on foliar stomata of *Vicia faba* L. Advances in Plant Sciences. 1996;**5**(2):483-495

[50] Khan AA, Ansari M, Talat K, Neelu K, Khursheed T, Kalra N. Studies on the effect of 6-benzyl aminopurine on meiosis in feba bean (*Vicia faba* L.). Advances in Plant Sciences. 2000;**11**(2):121-125

[51] Soliman AS. Studies on ssed germination and seedling growth of some African Acacias [thesis]. Institute of African Research and Studies, Cairo Univ.; 2004. pp. 6-11

[52] Bartsch M, Bednarek P, Vivancos PD, Schneider B, von Roepenack-Lahaye E, Foyer CH, et al. Accumulation of isochorismate-derived 2,3-dihydroxybenzoic 3-O-β-D-xyloside in Arabidopsis resistance to pathogens and aging of leaves. The Journal of Biological Chemistry. 2010;**285**(33):25654-25665

[53] Lumba S, Cutler S, McCourt P. Plant nuclear hormone receptors:

A role for small molecules in protein-protein interactions. Annual Review of Cell and Developmental Biology. 2010;**26**:445-469

[54] Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, et al. Crosstalk between abiotic and biotic stress responses: A current view from the points of convergence in the stress signaling networks. Current Opinion in Plant Biology. 2006;**9**(4):436-442

[55] Awan K, Khurshid MY, Mehmood A. Plant growth regulators and their role in abiotic stress management Fahad. The International Journal of Innovative Biosciences Research. 2017;**1**(1):9-22

[56] Khan MA, Ungar IA. Alleviation of seed dormancy in the desert forb *Zygophyllum simplex* L. from Pakistan. Annals of Botany. 1997;**80**:395-400

[57] Atici O, Agar G, Battal P. Changes in phytohormone contents in chickpea seeds germinating under lead or zink stress. Biologia Plantarum. 2005;**49**:215-222

[58] Nikolic R, Mitic N, Miletic R, Neskovic M. Effects of cytokinins on in vitro seed germination and early seedling morphogenesis in *Lotus corniculatus* L. Journal of Plant Growth Regulation. 2006;**25**:187-194

[59] Peleg Z, Blumwald E. Hormone balance and abiotic stress tolerance in crop plants. Current Opinion in Plant Biology. 2011;**14**:290-295

[60] Falkowska M, Pietryczuk A, Piotrowska A, Bajguz A, Grygoruk A, Czerpak R. The effect of gibberellic acid (GA3) on growth, metal biosorption and metabolism of the green algae chlorella vulgaris (Chlorophyceae) Beijerinck exposed to cadmium and lead stress. Polish Journal of Environmental Studies. 2011;**20**(1):53-59

[61] Tuna AL, Kaya C, Dikilitas M, Higgs D. The combined effects of gibberellic acid and salinity on some antioxidant enzyme activities, plant growth parameters and nutritional status in maize plants. Environmental and Experimental Botany. 2008;**62**(1):1-9

[62] Ghanem M, Hichri I, Smigocki A, Albacete A, Fauconnier M, Diatloff E, et al. Root-targeted biotechnology to mediate hormonal signaling and improve crop stress tolerance. Plant Cell Reports. 2011;**30**:807-823

[63] Atanasova L, Pissarska MG, Popov GS, Georgiev GI. Growth and endogenous cytokinins of juniper shoots as affected by high metal concentrations. Biologia Plantarum. 2004;**48**:157

[64] Bajguz A. Brassinosteroid enhanced the level of abscisic acid in Chlorella vulgaris subjected to short-term heat stress. Journal of Plant Physiology. 2009;**166**:882

[65] Sadak MS, Dawood MG. Role of ascorbic acid and α tocopherol in alleviating salinity stress on flax plant (*Linum usitatissimum* L.). Journal of Stress Physiology & Biochemistry. 2014;**10**(1):93-111

[66] Rajanandh MG, Satishkumar MN, Elango K, Suresh B. *Moringa oleifera* Lam. a herbal medicine for hyperlipidemia: A preclinical report. Asian Pacific Journal of Tropical Disease. 2012;**2**:S790-S795

[67] Moussa HR, Hassan MA-E. Growth enhancers to mitigate salinity stress in *Vicia faba*. International Journal of Vegetable Science. 2016;**22**(3):243-250

[68] Rady MM, Abd El-Mageed TA, Abdurrahman HA, Mahdi AH. Humic acid application improves field performance of cotton (*Gossypium barbadense* L.) under saline conditions. Journal of Animal and Plant Sciences. 2016;**26**(2):487-493

[69] Battacharya D, Babgohari MZ, Rathor P, Prithiviraj B. Seaweed extracts as biostimulants in horticulture. Scientia Horticulturae. 2015;**196**:39-48

[70] Azra Y, Basra SMA, Muhammad F, Hafeez ur R, Nazim H, Habib RA. Exogenous application of moringa leaf extract modulates the antioxidant enzyme system to improve wheat performance under saline conditions. Plant Growth Regulation. 2013;**69**:225-233

[71] Aslam M, Sultana B, Anwar F, Munir H. Foliar spray of selected plant growth regulators affected the biochemical and antioxidant attributes of spinach in a field experiment. Turkish Journal of Agriculture and Forestry. 2015;**39**:1-10

[72] Soliman AS, Shanan NT. The role of natural exogenous foliar applications in alleviating salinity stress in *Lagerstroemia indica* L. seedlings. Journal of Applied Horticulture. 2017;**19**(1):35-45

[73] Kang SM, Khan A, Waqas M, You YH, Kim JH, Kim JG, et al. Plant growth-promoting rhizobacteria reduce adverse effects of salinity and osmotic stress by regulating phytohormones and antioxidants in *Cucumis sativus*. Journal of Plant Interactions. 2014;**9**(1):673-682

[74] Hussein MMM, Haggag AA. Effect of irrigation intervals and salt concentrations on the growth and chemical composition of *Asclepias curassavica* L. Annals of Agricultural Sciences. 2003;**48**(1):307-327

[75] Gupta G, Parihar SS, Ahirwar NK, Snehi SK, Singh V. Plant growth promoting rhizobacteria (PGPR): Current and future prospects for development of sustainable agriculture. Journal of Microbial and Biochemical Technology. 2015;7:096-102

[76] Soliman AS, Sakr WRA. Improving tolerance of *Vachellia farnesiana* plants to irrigation water salinity by using bio-inocula under sandy soil conditions. American-Eurasian Journal of Agricultural & Environmental Sciences. 2017;**17**(1):01-21

[77] Soliman AS. Effect of Rhizobia isolated from some Acacias of on growth of *Acacia nilotica* under some stress conditions in North Africa [thesis]. Department of Natural Resources, Institute of African Research and Studies, Cairo University; 2008

[78] Aroca R. Ruiz-Lozano JM Induction of plant tolerance to semi-arid environments by beneficial soil microorganisms—A review. Sustainable Agriculture Reviews. 2009;**2**:121-135

[79] Soliman AS, Morsy EM, Massoud ON. Tolerance of bio-fertilized *Delonix regia* seedlings to irrigation intervals. Journal of Horticulture and Forestry. 2015;**6**(3):73-83

[80] Colla LM, Reinehr CO, Reichert C, Costa JAV. Production of biomass and nutracenutical compounds by *Spirulina platensis* under different temperature, nitrogen sources. Bioresource Technology. 2007;**98**:1489-1493

[81] Liu XM, Zhang FD, Zhang SQ, He XS, Fang R, Feng Z, et al. Effects of nano-ferric oxide on the growth and nutrients absorption of peanut. Plant Nutrition and Fertilizer Science. 2005;**11**:14-18

[82] Zhu H, Han J, Xiao JQ, Jin Y. Uptake, translocation and accumulation of manufactured iron oxide nanoparticles by pumpkin plants. Journal of Environmental Monitoring. 2008;**10**:713-717

[83] Soliman AS, El-feky SA, Darwish E. Alleviation of salt stress on *Moringa peregrina* using foliar application of nanofertilizers. Journal of Horticulture and Forestry. 2015;7(2):36-47

[84] Ghaffari H, Razmjoo J. Response of durum wheat to foliar application of varied sources and rates of iron fertilizers. Journal of Agricultural Science and Technology. 2015;**17**:321-331

[85] Babaei K, Sharifi RS, Pirzad A, Khalilzadeh R. Effects of bio fertilizer and nano Zn-Fe oxide on physiological traits, antioxidant enzymes activity and yield of wheat (*Triticum aestivum* L.) under salinity stress. Journal of Plant Interactions. 2017;**12**(1):381-389

Chapter 2

Kinetic Studies on Cell Growth

Punniavan Sakthiselvan, Setti Sudharsan Meenambiga and Ramasamy Madhumathi

Abstract

The kinetic model of cell growth is substantially capable to predict product formation. Mathematical models provide a strategy for solving problems encountered in fermentation process. A biochemical engineering approach to address this problem could be to develop a mathematical model which not only helps in the understanding of the system but also predicts various cultivation strategies to facilitate the optimization of a fermentation process, saving much of the time and cost for performing experiments. The presented overview indicates that many of the environmentally relevant aspects in growth kinetics are still waiting to be discovered, established, and exploited. A kinetic model that describes microbial growth, product formation and substrate consumption and the experimental data were fitted with modified logistic equation.

Keywords: cell growth, kinetics, fermentation, biomass, growth associated, product

1. Introduction

Cell growth implies increase in its mass and physical size controlled by physical, biological and chemical environments. Microbial growth is quantified by increase in the macromolecular and chemical constituents of the cell and growth pattern of each microbe is unique. Cell growth and cell division are inseparable for microbes as bacteria divide by binary fission, yeast cells by budding and viruses divide intracellularly [1]. Microbial growth during log phase is very important for the analysis of cells due to division by binary fission [2]. A typical mammalian cell growth is influenced by nutrient availability and thus a threshold cell size is required for DNA synthesis and mitosis [3]. Thus, each class of organisms have a different growth pattern based on their cell cycle and cell division. Understanding the growth kinetics of different classes of organisms forms the basis for fermentation process to achieve optimum product concentration.

Growth kinetics is an autocatalytic reaction which implies that the rate of growth is directly proportional to the concentration of cell. The cell concentration is measured by direct and indirect methods. Direct methods include measuring the cell mass concentration and cell number density by its dry weight, turbidity (optical density), plate counts etc. Whereas, indirect methods of measuring cell density are done by measuring the concentration of proteins, ATP or DNA content [4].

Batch growth kinetics of a microbe follows a growth curve with lag phase as the initial phase during which cells adapt to a new environment. Multiple lag phases occur if the media is supplemented with more than one sugar and such type of

growth is referred to as diauxic growth. Following the lag phase is the log phase in which the cell mass and cell number increases exponentially and then the depletion of nutrients starts which indicates the deceleration phase. The accumulation of toxic products results in deceleration phase after which stationary phase commences in which growth rate equals the death rate. The continuous growth kinetics accessed by a perpetual feeding process in which the growth is controlled by the concentration of the rate limiting nutrient [5].

Microbial growth kinetics explains the relationship between the specific growth rate of a microbe and its substrate concentration. Microbial growth kinetics largely depends on the laboratory culture conditions. In batch culture, microbial cell composition and its state change as a function of time and thus the rate of increase in biomass concentration was monitored [6]. Alternatively, in continuous culture the concentration of substrate is at equilibrium and the culture grows at stable physiological state which provides more precise and reproducible data [7, 8]. However, the constant growth conditions represent an artificial growth environment which does not explain many microbial kinetic phenomena. Thus, growth of microbial cells was performed under mixed substrates rather than single substrate to understand the growth kinetics of microorganisms in their natural environment [9].

The substrate such as nutrients (carbon and nitrogen sources), hormones and growth factors influence the growth pattern of microbial and mammalian cells. Substrate limited and substrate-sufficient growth would be observed on the basis of the relative availability of the substrate and the organisms utilize more substrate and energy under substrate sufficient conditions which in turn produces different patterns of product formation. A term describing the residual substrate concentration in Leudeking-Piret model was thus extended in the product formation kinetics [10].

Product of interest is traditionally achieved in the fermentation industry by metabolic engineering of few microorganisms which involves many genetic engineering techniques. The complexity of such genetic modifications and microbial metabolism due to various interconnected pathways urges the need to focus on developing mathematical models for identifying targets of metabolic engineering [11, 12].

Mathematical models are kinetic models which explain the relationship between rates and the concentration of reactants/products and allows to predict the rate of conversion of reactions in to products. This simulated model thus paved way for the optimal design of the operating conditions and operating design of the process for optimal product formation. Qualitative models were mostly used by researchers rather than quantitative models for gene expression systems as quantitative prediction of process parameters are complicated. However, with the advancements in experimental techniques of life sciences and using powerful computer technology, complex mathematical models were developed which is used for the design of various bioprocesses [13]. Industrial Biotechnology largely makes use of such mathematical models and saves time and resources with a clear understanding of strategies to optimize the product yield. Other potential uses of mathematical models include increasing the range of substrates, reduction of undesirable product formation and on the whole optimization of fermentation processes [14, 15].

Studying growth of a microorganism is the basis of biotechnological exploitation of microflora for production of desired product. Optimization of growth of microorganism in a particular media is desirable due to economical and availability of particular growth constituent in a region. Despite this, some microorganisms have specific requirement and they grow in a particular growth media.

The presented overview thus provides a knowledge on the fundamental basics of microbial growth kinetics and energetics which forms the basis for bio-engineering in optimizing, producing and purification of commercially novel products.

2. Growth kinetics

Classified based on the relationship between product synthesis and energy generation in the cell:

- Growth associated
- Non-growth associated
- Mixed-growth associated

2.1 Growth associated

Growth linked products are formed by growing cells and hence primary metabolites. **Figure 1** clearly shows that product is formed simultaneously with growth of cells. That is product concentration increases with cell concentration. The formation of growth associated product may be described by Eq. (1);

$$\frac{dP}{dt} = r_p = q_p X \tag{1}$$

where P = concentration of product
q_p = specific rate of product formation
X = biomass concentration.

2.2 Non-growth associated

They are formed by cells which are not metabolically active and hence are called secondary metabolites. **Figure 2** clearly shows that product formation is unrelated

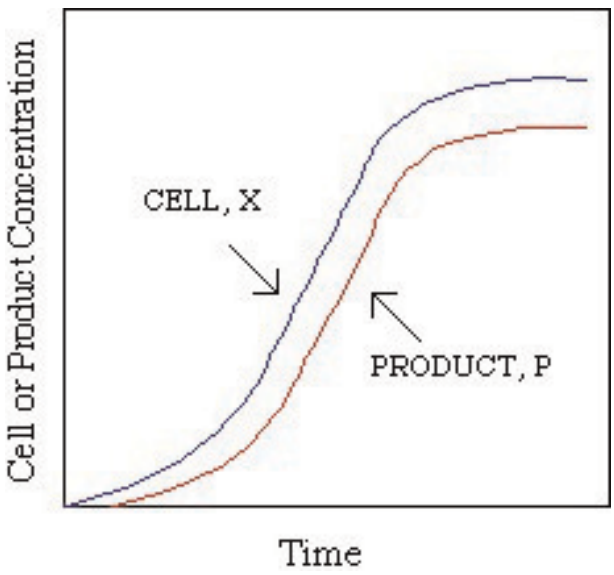

Figure 1.
Growth associated.

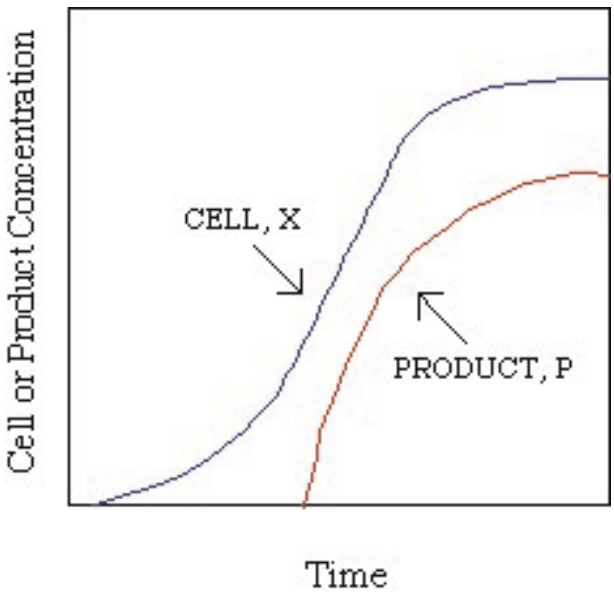

Figure 2.
Non-growth associated.

to growth rate but is a function of cell concentration. The formation of Non-growth associated product may be described by Eq. (2);

$$q_p = \beta = constant \tag{2}$$

2.3 Mixed-growth associated

The product formation from the microorganism depends on both growth and Non-growth associated. It takes place during growth and stationary phases. In **Figure 3**, product formation is a combination of growth rate and cell concentration. The formation of Mixed-growth associated product may be described by Eq. (3);

$$q_p = \alpha_\mu + \beta \tag{3}$$

2.4 Production kinetics

Microbial growth kinetics, i.e., the relationship between the specific growth rate (μ) of a microbial population and the substrate concentration (s), is an indispensable tool in all fields of microbiology, be it physiology, genetics, ecology, or biotechnology, and therefore it is an important part of the basic teaching of microbiology [16]. Unfortunately, the principles and definitions of growth kinetics are frequently presented as if they were firmly established in the 1940s and during the following "golden age" in the 1950s and 1960s the key publications are those of Monod. Monod, logistic, modified logistic model, and Leudeking-Piret models were used to describe the batch growth kinetics of cell. The Monod kinetic model is given as Eq. (4):

$$\mu = \frac{\mu_{max} S}{K_s + S} \tag{4}$$

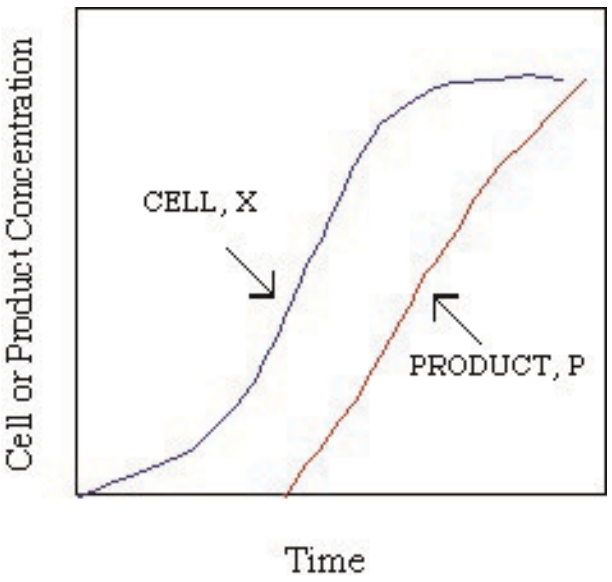

Figure 3.
Mixed growth associated.

where μ is the specific growth rate (h-1), S is substrate concentration (g/L) and K_S and μ_{max} are the Monod constant (g/L) and maximum specific growth rate, (h-1) respectively.

At the end of the lag phase, the growth of microorganisms is well acclimatized for its contemporary environment. Then the cells were multiplied hastily. The major active part of the cell growth curve which is called as the exponential (log) phase is used for the adjudication of kinetic parameters. The period of balanced growth that is the log phase, in which all components of a cell grow at the equivalent rate. Malthus model was also used for the cell growth behavior.

In Contois model, Michaelis constant is directly proportional to cell concentration and specific growth rate is inversely proportional to cell concentration which is described by Eq. (5). The Monod equation was also modified with the maintenance term which was incorporated in the Herbert model (Eq. (6)).

$$\mu = \frac{\mu_{max} S}{K_s \mathrm{X} + \mathrm{S}} \tag{5}$$

$$\mu = (\mu_{max} + m)\left(\frac{S}{K_s + \mathrm{S}}\right) - m \tag{6}$$

where X is cell mass concentration (g/L) and t is time (h). Separation of variables and integrating Eq. (4) yields Eq. (5). The above equations were used to enumerate the cell growth and product accumulation during the batch experiments [17]. The relationship between cell growth and product formation were identified by Leudeking-Piret kinetics.

Leudeking-Piret model (Eq. (7)) was used for kinetic analysis of cell production.

$$\frac{dp}{dt} = \alpha \frac{dx}{dt} + \beta x \tag{7}$$

where α and β are the associated and non-associated growth factor respectively. x and p show the concentration of dry cell weight (DCW) and product

concentration. The Logistic equation was used to analyze the exponential growth phase kinetics while Malthus kinetics was used to express the death phase kinetics (Eqs. (8) and (9)) [16, 18].

$$\frac{dx}{dt} = \mu_m \left(1 - \frac{x}{x_m}\right) x \tag{8}$$

$$\frac{dx}{dt} = \mu \cdot x \tag{9}$$

$$x(t) = \frac{x_0 \, exp\,(\mu_m \cdot t)}{\left[1 - \left(\frac{x_0}{x_m}\right)(1 - \, exp\,(\mu_m \cdot t))\right]} \tag{10}$$

$$1n\left(\frac{x}{x_0}\right)\mu \cdot t \tag{11}$$

where x_m, x_0 and μ_m are the initial DCW or biomass concentration, maximum biomass concentration and maximum specific growth rate of the microorganism, respectively. Also, tm is the required time (seed age) for maximum product concentration by the microorganism. According to Eq. (10), in order to estimate the value of the μm, a plot of $1n\frac{x}{x_m - x}$ against t will yield a straight line that the value of its the slope corresponds to μm and the intercept equals to $1n\left(\frac{x_m}{x_0} - 1\right)$. The substrate and product inhibitory effect on cell growth has been presented by Eq. (11), where x is biomass concentration with respect to time and x_0 is the initial biomass concentration.

$$1n\frac{x}{x_m - x} = \mu_m \cdot t - 1n\left(\frac{x_m}{x_0} - 1\right) \tag{12}$$

The growth pattern of micro-organism followed the modified Logistic model. Maximum cell concentration was obtained for sugarcane bagasse incubated for 48 h when compared to glucose as carbon source. The experimental values deviate slightly towards the end of stationary phase because the modified logistic equation used does not distinguish the decrease in cell density that normally occurs at the end of stationary phase [19]. Substituting Eqs. (8) and (10) into Eq. (7) and integrating, will yield Eq. (13).

$$\begin{aligned} p(t) = p_0 + \alpha x_0 \left\{ \frac{exp\,(\mu_m \cdot t)}{\left[1 - \left(\frac{x_0}{x_m}\right)(1 - \, exp\,(\mu_m \cdot t))\right]} - 1 \right\} \\ + \beta \frac{x_m}{\mu_m} 1n\left[1 - \left(\frac{x_0}{x_m}\right)(1 - \, exp\,(\mu_m \cdot t))\right] \end{aligned} \tag{13}$$

Eq. (13) can be rewritten as Eq. (14)

$$p(t) = p_0 + \alpha A(t) + \beta B(t) \tag{14}$$

The value of dx/dt is equal to zero and $x = x_m$ in the stationary phase. Using Eqs. (7) and (13), one can obtain:

$$\beta = \frac{\frac{dp}{dt}(st \cdot phase)}{x_m} \tag{15}$$

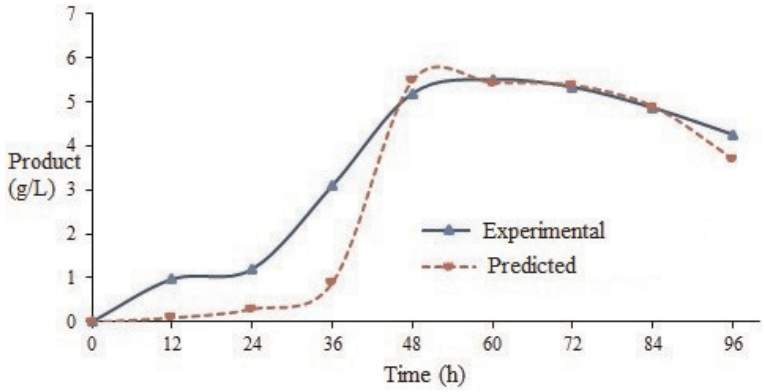

Figure 4.
The kinetic modeling of product production by Leudeking-Piret model using carbon source.

The value of x_m can be obtained from the experimental growth kinetic data and the value of parameter α was obtained from the slope of the linear plot of $p(t) - p_0 - \beta B$ against $A(t)$.

Eqs. (13) and (16) show the kinetic model of product production in the exponential growth phase and death phase, respectively.

$$p(t) = p_0 + \alpha x_0 \, exp\,(\mu \cdot t) + \beta \frac{x_0}{\mu} \, exp\,(\mu \cdot t) \qquad (16)$$
$$= p_0 + \alpha A(t) + \beta B(t)$$

The resulting graph obtained from kinetic modeling of product production by Leudeking-Piret model are shown in **Figure 4**. It is the combination of kinetic models for better agreement between experimental data and model predictions which are employed in cell growth and Product production. The product accumulation mostly adhered to growth-associated kinetic pattern. Matlab ver. 7.12 computer software was used to define the interpretation of growth kinetic parameters.

3. Conclusion

One of the very important practical applications of this model is the evaluation of the product formation kinetics. Mathematical models facilitate data analysis and provide a strategy for solving problems encountered in fermentations. Information on fermentation process kinetics is potentially valuable for the improvement of batch process performance. Finally, the product yields and substrate conversions are criteria with the main attention toward productivity.

Conflict of interest

This is an original work of the authors and it has not been submitted to any other open access publishers previously. Here we have declared that there is no conflict of interest.

Appendices and nomenclature

μ_{max}	maximum specific growth rate (h-1)
X_i	independent variables
X_o	initial biomass concentration (mg/ml)
X_{max}	maximum biomass concentration (mg/ml)
X	biomass concentration (mg/ml)
t	incubation time (h)
DCW	dry cell weight

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References

[1] Wang JD, Levin PA. Metabolism, cell growth and the bacterial cell cycle. Nature Reviews Microbiology. 2009;**7**(11):822

[2] Prosser JI, Tough AJ. Growth mechanisms and growth kinetics of filamentous microorganisms. Critical Reviews in Biotechnology. 1991;**10**(4): 253-274

[3] Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. Cancer Cell. 2007;**12**(1):9-22

[4] Harris CM, Kell DB. The estimation of microbial biomass. Biosensors. 1985;**1**(1): 17-84

[5] Okpokwasili GC, Nweke CO. Microbial growth and substrate utilization kinetics. African Journal of Biotechnology. 2006;**5**(4):305-317

[6] Kluyver AJ. Life's flexibility; microbial adaptation. In: The Microbes' Contribution to Biology. Cambridge, MA: Harvard University Press; 1956. p. 93

[7] Kovarova K, Käch A, Zehnder AJ, Egli T. Cultivation of *Escherichia coli* with mixtures of 3-phenylpropionic acid and glucose: Steady-state growth kinetics. Applied and environmental microbiology. 1997;**63**(7):2619-2624

[8] Senn H, Lendenmann U, Snozzi M, Hamer G, Egli T. The growth of *Escherichia coli* in glucose-limited chemostat cultures: A re-examination of the kinetics. Biochimica et Biophysica Acta (BBA)-General Subjects. 1994; **1201**(3):424-436

[9] Kovárová-Kovar K, Egli T. Growth kinetics of suspended microbial cells: From single-substrate-controlled growth to mixed-substrate kinetics. Microbiology and Molecular Biology Reviews. 1998; **62**(3):646-666

[10] Zeng AP. A kinetic model for product formation of microbial and mammalian cells. Biotechnology and Bioengineering. 1995;**46**(4):314-324

[11] Patil KR, Åkesson M, Nielsen J. Use of genome-scale microbial models for metabolic engineering. Current Opinion in Biotechnology. 2004;**15**(1):64-69

[12] Noack S, Wahl A, Qeli E, Wiechert W. Visualizing regulatory interactions in metabolic networks. BMC Biology. 2007; **5**(1):46

[13] Nielsen J, Villadsen J, Lidén G. Modeling of growth kinetics. In: Bioreaction Engineering Principles. Boston, MA: Springer; 2003

[14] Tyo KE, Kocharin K, Nielsen J. Toward design-based engineering of industrial microbes. Current Opinion in Microbiology. 2010;**13**(3):255-262

[15] Cvijovic M, Bordel S, Nielsen J. Mathematical models of cell factories: Moving towards the core of industrial biotechnology. Microbial Biotechnology. 2011;**4**(5):572-584

[16] Kovar KK, Egli T. Growth kinetics of suspended microbial cells: From single-substrate controlled growth to mixed-substrate kinetics. Microbiology and Molecular Biology Reviews. 1988;**62**: 646-666

[17] Najafpour GD. Growth kinetics. In: Biochemical Engineering and Biotechnology. Amsterdam: Elsevier; 2007. p. 81

[18] Bailey JE, Ollis DF. Biochemical Engineering Fundamentals. 2nd ed. New York: McGraw-Hill; 1986

[19] Wachenheim DE, Patterson JA, Ladisch MR. Analysis of the logistic function model: Derivation and applications specific to batch cultured microorganisms. Bioresource Technology. 2003;**86**:157-164

Chapter 3

Growth of Fungal Cells and the Production of Mycotoxins

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Abstract

Some filamentous fungi are able to grow in food and produce toxic metabolites. It occurs mainly in grains, cereals, oilseeds and some by-products. The growth of fungi in a particular food is governed largely by a series of physical and chemical parameters. The production of toxic metabolites is not confined to a single group of molds irrespective of whether they are grouped according to structure, ecology, or phylogenetic relationships. Mycotoxins can be carcinogenic and cause several harmful effects to both human and animal organisms, in addition to generating large economic losses. The major mycotoxins found in food are the aflatoxins, fumonisins, ochratoxins, patulin, zearalenone, and trichothecenes, generally stable at high temperatures and long storage periods. Considering the difficult prevention and control, international organizations for food safety establish safe levels of these toxins in food destined for both human and animal consumption. Good agricultural practices and control of temperature and moisture during storage are factors which contribute significantly to inhibit the production of mycotoxins. The use of some fungistatic products, such as essential oils and antioxidants, as well as physical, mechanical, chemical, or thermal processing, represents important methods to have the concentration of mycotoxins reduced in food.

Keywords: aflatoxins, ochratoxins, patulin, fumonisins, zearalenone

1. Introduction

Microorganisms constitute the main cause of deterioration and losses in food. Fungi can be mono- or multiple-cell organisms, mostly aerobic, which survive within a wide range of moisture, temperature, and pH. They inhabit nature freely and feed on the absorption of organic matter.

Their presence in food can be derived from the field, such as parasites, plant pathogen, and even coming from the soil or equipment used in the management of culture crops. In addition, they appear as storage microbiota and develop during the entire storage process, which may lead to great physical-chemical and sensory losses in food products, in addition to the production of mycotoxins.

Mycotoxins are substances secreted by the secondary metabolism of filamentous fungi, which are produced by certain fungus lineages and in particularly favorable conditions. A few hundreds different mycotoxins are known, some characterized by their antibiotic potential and others extremely toxic to men and animals. This chapter will present the fungi growth conditions to produce mycotoxins, the major mycotoxins occurring in food, levels of toxicity, favorable conditions to excretion, and control measures regarding their production.

2. Fungi

Fungi are able to grow in practically all ecological niches; however, they can be found prevailing particularly in dead organic matters present in the soil. They include eukaryotic organisms commonly known as yeasts, which normally grow in the form of single cells, and molds, which grow by forming ramified chains called hyphae. Even though most fungi are harmless to human beings, the exposition to specific lineages and their metabolites may result in some clinical manifestations in men and other animals.

a. Infections or diseases derived from the invasion of living tissue. The growth of a fungus on the top of or inside a body is named mycosis. Mycoses can have varied severity, encompassing from relatively benign and superficial infections to severe diseases that threaten life.

b. Hypersensitivity reactions. Some fungi promote an immune response which can result in allergic reactions after exposition to a specific fungus antigen. Exposition to fungi, either by developing in a host or in the environment, may cause the development of allergic symptoms in the case of a re-exposition. For example, *Aspergillus s*pp., a common saprophyte, often found in nature as a filamentous fungus from leaves, corresponds to a potent, common allergen, which often triggers asthma and other hypersensitivity reactions.

c. Mycotoxicosis. It is an intoxication resulting from ingestion of food or feed containing toxic metabolites, that is, mycotoxins [1].

The major toxigenic strains of interest for the public health belong to the genera *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. It is important to highlight that not all fungi produce mycotoxins as well as that a single fungus species can produce many secondary toxic compounds. The presence of a mycotoxin in food is necessarily conditioned to fungus development, but it does not mean that a product without any fungi could not contain mycotoxins [2]. A cereal stored under poor conditions of temperature and moisture provides a favorable medium for fungus development

and the production of mycotoxins. Once a mycotoxin is produced, even upon the destruction of the fungus biomass, through a drying process, for example, the metabolites excreted remain in the product.

As heterotrophs, fungi require organic compounds for both synthesis of biomass (anabolic metabolism) and production of energy to drive these reactions (catabolic metabolism). These aspects are referred to as primary metabolism. The secondary metabolisms are distinct from primary metabolism in so far as they occur optimally after a phase of a balanced growth and are often, but not always, associated with morphogenetic changes such as sporulation; the production of particular secondary metabolites is usually restricted to a small number of species and may be species, or even strain, specific; it has not generally been possible to rationalize the biological function of secondary metabolites, although some are very active against microorganisms (antibiotics), plants (phytotoxins), or animals (mycotoxins). Although secondary metabolites in general, and mycotoxins specifically, do not form a neat and a recognizable group of organic structures, they can be classified in terms of the biosynthetic pathways leading to their production. This is so because the processes of primary and secondary metabolism are linked by a relatively small number of simple intermediates such as acetyl coenzyme A, mevalonic acid, and amino acids [3–5].

3. Fungal growth in food

The growth of fungi in a particular food is governed largely by a series of physical and chemical parameters, and definition of these can assist greatly in assessing the food's stability. The factors which govern spoilage are physical and chemical, and there are eight principal factors: water activity; hydrogen ion concentration; temperature, of both processing and storage; gas tension, specifically of oxygen and carbon dioxide; consistency; nutrient status; specific solute effects; and preservatives [6].

In general, fungal deterioration stands out under conditions in which bacterial deterioration is controlled, either by low water activity (aw), pH, temperature, and/or the presence of inhibitory agents. Raw material quality and contamination of the production environment will directly interfere with the initial contamination of the products. The processing and storage parameters will influence on time to the appearance of visible fungal colonies and, therefore, the shelf life of a food product [6, 7].

The deterioration of food by filamentous fungi starts with contamination of the product by fungal spores originating from the environment. When intrinsic parameters, such as water activity (aw) and pH, as well as temperature, are favorable, the spores will germinate and form a visible mycelium, deteriorating the product [8, 9].

Temperature and aw are recognized as the most important parameters for determining fungi cell growth, but pH also influences that development. The external pH value influences not only fungal growth rate but also metabolism. *Aspergillus flavus* isolates produce more aflatoxins when the external pH becomes increasingly acidic. In the case of the cereal pathogen *Fusarium graminearum*, trichothecene production is induced only under acidic pH conditions [10].

The moisture content of grains and other dried foods is such that there is seldom any problem with the growth of bacteria and yeasts, but there are frequent problems with the growth of molds (fungi). Unless the aw is reduced to below approximately 0.7, molds will grow on any food, and as the relative humidity in the humid tropics is generally more than 70%, almost all dry foods will become moldy when stored in the humid tropics unless the moisture content is reduced to an aw of less than 0.70, followed by storage that will protect that food from absorbing moisture from the high-humidity environment [11–14].

4. Mycotoxins

Mycotoxins have been known for a very long time, but they only became more intensively studied after an incident occurred in 1960 in England, involving the death of 100,000 birds fed on feed contaminated with fungus *Aspergillus flavus*.

Mycotoxins are produced mainly by mycelial structures present in filamentous fungi. Even though their function for produced lineages is yet to be clarified, mycotoxins are secondary metabolites that apparently do not present a biochemical meaning to fungus growth and development [7, 15].

According to the Food and Agriculture Organization (FAO), it is estimated that the contamination of food products by fungi and their toxic metabolites generates qualitative and quantitative losses for around 25% of the agricultural food production worldwide, occurring majorly in regions of tropical and subtropical climate, where higher temperature and moisture favor microbial proliferation [16, 17].

Primary metabolites of fungi, such as of other organisms, are essential to growth, while secondary ones are formed during the final exponential growth phase and have not a clear significance to the growth or metabolism of the organism [1–3].

In general, these metabolites appear to be formed whenever large amounts of primary metabolites precursors, such as amino acids, acetate, and pyruvate, among others, are accumulated. The synthesis of mycotoxins represents a way for fungi to reduce the amount of precursors, which are not required to metabolism [1–3].

They are constituted by a large variety of chemical assembles, which provides them with several biological activities, classified according to the toxicity level exerted on human and animal organisms [2] possibly with carcinogenic, mutagenic, teratogenic, cytotoxic, neurotoxic, nephrotoxic, immunosuppressant, and estrogen effects. However, its toxicity largely depends on the amount ingested, time of exposition, and possible synergy with the ingestion of many different mycotoxins simultaneously, in addition to individual physiological conditions [4].

Mycotoxin ingestion can produce both acute and chronic toxicities. Acute is characterized by a rapid onset and an obvious toxic response including rapid death. Chronic is resulting from low-dose exposure to mycotoxins over a long period of time, with toxic responses including cancers such as hepatocellular carcinoma [18, 19].

The International Agency for Research on Cancer (IARC) in Lyon (France)—through its IARC Monographs program—has performed the carcinogenic hazard assessment of some mycotoxins in humans, on the basis of epidemiological data, studies of cancer in experimental animals, and mechanistic studies. There are five groups classified according to the scientific evidence for their carcinogenicity: Group 1, carcinogenic to humans; Group 2A, probably carcinogenic to humans; Group 2B, possibly carcinogenic to humans; Group 3, not classifiable as to its carcinogenicity to humans; and Group 4, probably not carcinogenic to humans. Carcinogenic effects and related mechanisms of some mycotoxins (e.g., aflatoxins) are well-known. However, for some other important mycotoxins (e.g., OTA, FUM B1, and FUM B2), there is a need for continued research on understanding these mechanisms [20–24].

4.1 Aflatoxins

Many types of aflatoxin (14 or more) occur in nature, but only four of them are particularly dangerous to humans and animals. Aflatoxins are mainly produced by species of *Aspergillus flavus* and *Aspergillus parasiticus* and classified in aflatoxins B1 and B2 and G1 and G2. Their name derives from the fluorescence emitted after absorption of ultraviolet light at 365 nm (B, blue; and G, green). They are characteristically heat-resistant and bear the process of sterilization with a structure

remaining unaltered for long periods of storage. Their chemical structure allows good solubility in organic solvents and are insoluble in water proving sensitive in alkaline medium, which decreased their toxicity [22]. The group of aflatoxins is considered by the IARC as belonging to Group 1—cancer-causing substances to men [21].

Mycotoxin B1 is pointed out as the most toxic in the group and can generate metabolites in the organism of mammals (M1). Aflatoxin M1, generated by B1 metabolism, is transported to milk at a proportion of 1% of the total ingested and can also be found in animal tissues in which there is a high exposition to this toxin. Subsequently, humans can ingest this aflatoxin through breast milk, milk, and milk-derived products, especially in areas where grains and cereals of poor quality are used to feed animals [17–23].

Birds are the most sensitive animals to aflatoxins, and whenever ingested in contaminated feed, they present fast absorption through the gastrointestinal tract. The effects include damages to the liver, harm to the productivity and reproductive efficiency, decreased production of eggs, lower quality of egg shell, lower quality of carcass, and increased susceptibility to diseases. Pigs are, somehow, less sensitive than birds. Aflatoxin is also hepatotoxic to these animals, and its chronic effects are largely attributed to damage to the liver. In cattle, the primary symptom is reduction in weight gain. In addition, milk production is reduced [25–27].

Aflatoxins occur mainly in products such as peanut and nuts, maize, and cottonseed, among others, which is associated with pre-crop contamination. Cultures such as coffee, rice, and spices can also be contaminated by these toxins post-crop [26].

Many countries introduced legislation specific to mycotoxins. Most of these legislation rules are regarding aflatoxins, alkaloids of ergot, deoxynivalenol, and ochratoxins. Even though legal measures are yet to be uniformed at a worldwide level, the Codex Alimentarius Commission is gathering efforts to establish international guidelines regarding levels of mycotoxins. For aflatoxins, the Food and Drug Administration established the maximum limit of 20 ppb to maize, peanut, cotton bran, and other food and ingredients for animals [27–31].

4.2 Ochratoxin A

The major toxins produced by *Penicillium verrucosum*, *Aspergillus ochraceus*, and *Aspergillus carbonarius* are ochratoxin A, ochratoxin B, and ochratoxin C. Among these toxins, ochratoxin A is considered the most toxic and, according to the IARC, a possible cancer-causing substance to humans (category 2B). Recent researches conducted over the past 6 years related to ochratoxin toxicity encompassed the identification of factors involved in carcinogenesis and provided strong evidence to a reclassification of the Group 2B into the Group 2A (probably carcinogenic to human beings) [1, 4, 20].

Among the species of *Penicillium*, *P. verrucosum* is the major source of ochratoxin A and the most common species in countries of temperate, cold climate, while *A. ochraceus*, *A. carbonarius*, and other species from the Group A niger are the most common in tropical, hot climates. Another species of *Penicillium* produced from ochratoxin A is *P. nordicum*. *P. verrucosum* is especially associated with stored cereals, that is, post-crop fungi. This mycotoxin is often found in animal feed and food as wheat, rye, coffee, nuts, and, at a lower degree, grapes, raisins, wine, or products derived from pork. There have been reports of this mycotoxin detected in blood and milk breast of individuals exposed to its ingestion [1–4]. The levels can accumulate in the tissues of the body and fluids of human beings or animals who consume contaminated food. Evidence shows that ochratoxin A is slowly eliminated from the body [17, 25].

The structure of these toxins is derived from L-phenylalanine, which makes it a potent inhibitor of the enzyme phenylalanine-RNAt synthase, responsible for the synthesis of proteins of high turnover rich in phenylalanine—a functional role for kidney homeostasis. In addition, it interferes in the lipid peroxidation causing damages to the DNA and oxidative stress. Therefore, it is suspected that ochratoxin A is one of the cancer-causing agents in the urinary tract as well as related to the damages to kidneys occurred in Eastern Europe. Researches indicate that practically all Europeans have some ochratoxin concentration in their blood. Human exposition to ochratoxin occurs primarily from brown bread. In some parts of Europe, the most significant exposition derives from the consumption of animal products, especially those formulated based on pig blood [32, 33].

Considering the toxic effects of ochratoxins, a tolerable weekly intake (TWI) of 120 ng/kg of body weight (pv) was established by the European Food Safety Authority (EFSA). The meeting of the Committee of Specialists on Agricultural Contaminants in food (European Commission, DG Health, and food safety) has been considering establishing limits to herbal teas, infusions, and baking [32]. Even though ochratoxins B and C are hepatotoxic, immunotoxic, teratogenic, and genotoxic, maximum tolerable limits are yet to be established regarding these toxins [34, 35].

4.3 Patulin

Patulin (polyketide lactone 4-hydroxy-4H-furo (3.2c) pyran-2 (6H)-one) is a secondary metabolite produced by several species of *Penicillium*, *Aspergillus*, and *Byssochlamys* in conditions of high activity of water (0.95–0.99) and temperature of 0–31°C. Food which are more susceptible to contamination by patulin in human diet are apples and by-products (puree and juices). Even though contamination with patulin is mainly associated with areas of contaminated tissue, it can penetrate around 1 cm in healthy regions of the fruit [1].

Patulin has been reported as mutagenic, neurotoxic, immunotoxic, and genotoxic and to cause gastrointestinal damages in rodents. There is also some concern that similar effects may occur in humans through a long-term consumption of food and beverage contaminated with this mycotoxin. The IARC classified patulin as Category 3, non-classifiable regarding its carcinogenicity to human beings. Because of its toxicity, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) established a maximum tolerable limit for daily intake (PMTDI) for patulin of 0.4 μg/kg of body weight [34]. The Codex Alimentarius established a maximum level for patulin of 50 μg/kg in apple juice, and the European Union (EU) adopted a maximum level of 50 μg/kg 1 for juices, beverages, and fermented milk products containing apple juice, 25 μg/kg in solid products containing apple, and 10 μg/kg in apple-based products as well as baby food. Although some limits have been established, some countries, such as Pakistan, do not have any specific legislation for this toxin [36].

4.4 Trichothecenes

Trichothecenes are a group of secondary metabolites produced by fungi belonging to the genus *Stachybotrys* and mainly *Fusarium*, in which *F. graminearum* and *F. culmorum* are the most important. *Fusarium graminearum* grows greatly at a temperature of 25°C and activity of water above 0.88, while *F. culmorum* grows well at 21°C and activity of water above 0.87 [1].

The group of trichothecenes is composed of over 200 mycotoxins and carries this name because of their chemical structure constituted of a ring with tetracyclic skeleton 12,13-epoxitrichothecenes. They also present varied ligand assembles, which

provides toxicity. These toxins are classified in type A, in which toxins T-2, HT-2, 15-monoacetoxyscirpenol (15-MAS), and diacetoxyscirpenol (DAS) are found, and type B, in which deoxynivalenol (DON) occurs. Deoxynivalenol is an epoxy-sesquiterpenoid which occurs naturally combined with 3-acetyl-deoxynivalenol (3AcDON) and 15-acetyl-deoxynivalenol (15AcDON), which increases DON toxicity. According to the IARC, DON is classified in level 3, that is, it does not represent a risk of cancer induction, although co-occurrence with aflatoxin may increase aflatoxin carcinogenicity. DON can also coexist combined with zearalenone [1, 2, 37].

Despite the existence of a relatively large amount of different trichothecenes, their natural occurrence in food and feed is mainly related to nivalenol, deoxynivalenol, toxin T-2, diacetoxyscirpenol, and less often furarenone-X, toxin HT-2, and neosolaniol.

DON is probably the most largely distributed mycotoxin in food and feed often detected in wheat, barley, rye, malt, oat, maize, and consequently products derived from these cereals, such as flours and beers, and less often rice, sorghum, and triticale. It is a heat-resistant toxin which is not altered during food processing, in addition to being stable over long storage periods [37, 38].

Discovered in the early 1970s, DON is also popularly known as vomitoxin for its acute effect on the ingestion of high doses causing symptoms similar to enterotoxins of *Staphylococcus aureus* and *Bacillus cereus*, such as nausea, vomit, abdominal pains, diarrhea, leukopenia, bleeding, and even death in humans and animals [37, 38].

Pig is the most sensitive animal to DON, and the chronic effects of ingestion of contaminated feed result in reduced weight gain and growth, infertility, including the birth of animals with malformation, and miscarriage. Birds are more tolerant than pig, and the effects of intoxication are lower quality and weight of the eggs produced. Cattle are more tolerant, possibly due to the toxin degradation in secondary metabolites in the rumen. The effects in cattle include lower feed consumption and conception rate in addition to reduced milk production [2, 37].

The Codex Alimentarius Commission (CAC) establishes the maximum level of 2 μg/kg for DON for wheat, maize, and barley. The European Union, in turn, proposes 0.75 μg/kg for cereals and flour and 0.2 μg/kg for wheat germ [38].

4.5 Zearalenone

Also known as toxin F-2, it is an estrogen produced mainly by *F. graminearum*, *F. culmorum*, and *F. sporotrichioides*. Toxigenic strains of *Fusarium* can develop in soft climate and the optimum temperature to produce zearalenone is until 28°C. It is commonly found in several cereals, such as wheat, barley, sorghum, and mainly maize [6].

Zearalenone is a lactone of beta-resorcylic macrocyclic acid with a structure similar to 7ß-estradiol, main hormone produced in female human ovary. Zearalenones are considered micro-estrogenic due to their capacity to hamper the effect of steroid hormones interfering in human and animal reproductive capacities. It also influences the production of testosterone, progesterone, and estradiol. Zearalenone is able to imitate the activity of estrogen in the reproductive tract, including accessory glands, such as the prostate [40–42].

It causes hyperestrogenism in pig, whose symptoms are swelling and redness of the vulva and hyperdevelopment of the uterus and mammary glands. In addition to present significant effects on the increase of endometrial secretions and synthesis of uterine proteins and higher weight of reproductive organs [31].

Birds are more resistant to intoxication by zearalenone, but the many associations of fusariotoxin with other mycotoxins can result in severe losses. The production of zearalenone may occur either in the field or post-crop in inadequate storage

conditions (high moisture). The detection of this mycotoxin in bird feed has been considered a biomarker for other toxins belonging to the genus *Fusarium* [31].

Despite some evidence, the IARC assessed the carcinogenicity of zearalenone and found it to be a possible cancer-causing substance to humans. Zearalenone residues do not seem to be an issue after consumed.

4.6 Fumonisins

They were discovered in 1988 and described as fumonisins B1, B2, and B3, in which B1 occurs more frequently. However, fumonisins constitute a group encompassing over 16 substances already identified, called B1 (FB1, FB2, FB3, and FB4), A1, A2, A3, AK1, C1, C3, C4, P1, P2, P3, PH1a, and PH1b. They are highly water-soluble unlike other mycotoxins and do not have an aromatic structure or a single chromophore to analytically facilitate its identification, therefore being difficult to identify through ultraviolet spectrum [25].

These substances are produced by several species of the genus *Fusarium*, especially by *Fusarium verticillioides* (previously classified as *F. moniliforme*), *F. proliferatum*, and *F. nygamai*, in addition to *Alternaria alternata*. Other species, such as *F. anthophilum*, *F. dlamini*, *F. napiforme*, *F. subglutinans*, *F. polyphialidicum*, and *F. oxysporum*, also have been included in the group of these mycotoxin products [25, 38].

Fumonisins have been found as a common contaminant in maize-based food and feed. When ingested, fumonisins present low bioavailability and are rapidly metabolized and excreted. The carcinogenic nature of fumonisins does not seem to involve an interaction with DNA. Their mode of action is related to their toxicity in the interference of the biosynthesis of sphingolipid, which are very important to maintain the integrity of the cell membrane, regulation of receptors of cell surface, ion pump, regulation of growth factors, and other vital systems for the functioning and survival of the cell. In addition, fumonisins are potent immunosuppressant agents and can enhance the susceptibility to diseases [38–44].

These toxins cause many diseases in animals, such as leukoencephalomalacia (LEME) in horses and pulmonary edema in pigs. LEME is a noninfectious, highly fatal disease which affects the central nervous system of horses and other equines with a large distribution worldwide and considered a disease derived from regions of temperate, tropical climate. LEME involves metabolic alterations that produce the softening of the white substance of the encephalon as well as its liquefaction, which occurs due to a mycotoxin present in the feed. The disease occurs because of the need to supplement horse diet with grains of maize or feed containing them in their formulation due to the lack of fodder in pastures [43].

Even though their effects on human beings are difficult to determine, fumonisins have been statistically associated with high occurrence of esophageal cancer in South Africa and liver cancer in certain endemic areas in China. Based on toxicological evidence, the IARC declared the toxins of *F. moniliforme* as potentially carcinogenic to humans (Class 2B) [44].

4.7 Modified mycotoxins

The major mycotoxins in food (aflatoxins, ochratoxins, patulin, deoxynivalenol, zearalenones, and fumonisins) occur freely and coexist with modified mycotoxins. The term "mask mycotoxin" was used for the first time for mycotoxin M1, derived from the hydroxylation of aflatoxin B1, excreted in the milk of animals which consume contaminated feed with aflatoxin B1. In the mid 1980s, a new compound derived from zearalenone was found to be involved in cases of smycotoxicosi and no correlated to the mycotoxins found in the food matrix in question [45].

Recently, with the modernization of the means to detect the toxins, such as high-performance liquid chromatography associated with mass spectrometry, many other compounds derived from mycotoxins were discovered, and the term "mask mycotoxin" made way to the name of modified mycotoxins. This denomination classifies all substance derived from free mycotoxin, that is, all toxic substance originated by the secondary metabolism of fungi, which through a biological process (human, animal metabolism or even through the defense mechanism of plants) or technological process (food processing), have their structure altered as well as polarity, solubility, and molecular mass, originating a new substance with characteristic toxicity or able to reinforce the damages caused by parental mycotoxin [45, 46].

Some examples of modified mycotoxins are N-(1-deoxy-D-fructos-1-yl)-fumonisin B1 (NDF-FB1) and N-(carboxymethyl)-fumonisin B1 (NCM-FB1) formed in a Maillard reaction between fumonisins and reducing sugars and reaction of reduction occurring in DON when animal feed is treated with sodium bisulfite generating sulfonated DON [46].

5. Prevention and control of mycotoxins

Cereals, grains, and oilseed are often infected by insects and toxigenic fungi when still in the field interfering directly in the quality and productivity of food. Controlling fungi infestations is not an easy task for involving climatic and environmental issues which frequently cannot be controlled by men. Therefore, it is crucial to disseminate and implement techniques for good agricultural practices, indispensable to minimize problems related to the production of mycotoxins and quality of the food in the field. Some of these techniques involve choosing the variety to be cultivated by preferring lineages that are more resistant to attacks of plagues and microorganisms, good soil preparation, and turnover of cultures. It is also important to rationally employ agricultural pesticides by replacing them with sustainable techniques for plague control whenever possible, such as biological products, oils, and natural extracts, seeking to protect the cultivation and the environment [47].

The crop at the correct maturation point and the regulation of agricultural implements to soften mechanical damages to beans and grains are factors which combined with good storage practice can reduce fungus infestations in food products. The main storage practices encompass the improvement of the products received by removing impurities derived from the field, the control of moisture through drying process at recommended levels, good ventilation, cleansing of the storage location, and control of insects and rodents, in addition to a system to relative air humidity.

In addition to good agricultural and storage practices, some strategies for the detoxification of food and feed contaminated with mycotoxins have emerged as an effort to reduce or eliminate their toxic effects through chemical, physical, and biological methods. Some of them involve the application of gamma irradiation, ozone (O_3), and some microbial strains and fungus parasites able to inhibit the production or decrease the toxicity of some secondary metabolites like *Streptomyces rimosusand* and *Gliocladium roseum*, respectively. These methods are essential to improve food safety, prevent economic losses, and retrieve contaminated products [48, 49].

6. Conclusion

Mycotoxins are a group of fungal secondary metabolites, and their production is influenced by both the genotype of the organism and the physicochemical

environment in which it is growing. Even if a strain of mold has the genetic potential to produce a particular mycotoxin, the level of production will be influenced by the nutrients available. Even when the nutritional requirements are suitable for mycotoxin biosynthesis, physical parameters, such as temperature and water activity, will influence production. In nature there are many other factors interacting with the growth and metabolism of a mold. There may be, for example, antimicrobial agents produced by other microorganisms, by the plant on which the mold is growing, or added as biocides during crop husbandry. Mycotoxins have attracted worldwide attention not only because of their perceived impact on human health but also because of the economic losses accruing from contaminated foods. Mycotoxins have been extensively studied as well as their impact on human health. It is clear that food contaminated with toxic substances are not proper for either human or animal consumption. Considering that mycotoxins are natural contaminants and practically impossible to be completely eliminated from food, international food safety organizations provide guidance on the serious risks of mycotoxins to human health by updating and establishing safe levels of ingestion for these toxins. As a short-term solution, methods of prevention and food detoxification have been offered to producers aiming at providing means to enlarge the availability of safe food to the population worldwide.

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References

[1] Oliveira KAM, Vieira ENR. Microbiologia de Alimentos: Qualidade e Segurança na Produção e Consumo. Viçosa: UFV; 2003

[2] Iamanaka TB, Oliveira SI, Taniwaki HM. Micotoxinas em Alimentos. In: Anais da Academia Pernambucana de Ciência Agronômica. Vol. 7. 2010. pp. 138-161

[3] Smith D, Onoins AHS. The Preservation of Maintenance of Living Fungi. England: CAB; 1983. p. 51

[4] Jennings DH, Lysec G. Fungal Biology—Understanding the Fungal Lifestyle. USA: Springer; 1999. p. 166

[5] Smith JE, Moss MO. Mycotoxins—Formation, Analysis and Significance. Great Britain: John Wiley & Sons Ltd; 1985. p. 148

[6] Pitt JI, Hocking AD. Fungi and Food Spoilage. 3rd ed. Australia: Springer; 2009. p. 519

[7] Jay J. Microbiologia de Alimentos. 6th ed. Porto Alegre: Artmed; 2005

[8] Dagnas S, Membré JM. Predicting and preventing mold spoilage of food products. Journal of Food Protection. 2013;**76**:538-551

[9] Gougouli M, Kalantzi K, Beletsiotis E, Koutsoumanis KP. Development and application of predictive models for fungal growth as tools to improve quality control in yogurt production. Food Microbiology. 2011;**28**:1453-1462. DOI: 10.1016/j.fm.2011.07.006

[10] Coton E, Leguerinel I. Ecology of bacteria and fungi in foods. In: Batt CA, Robinson RK, editors. Encyclopedia of Food Microbiology. USA: Elsevier Science; 2014. pp. 577-586

[11] Kebede H, Abbas HK, Fisher DK, Bellaloui N. Relationship between aflatoxin contamination and physiological responses of corn plants under drought and heat stress. Toxins. 2012;**11**:1385-1403. DOI: 10.3390/toxins4111385

[12] Akbar A, Magan N. The impact of water and temperature interactions on lag phase, growth and potential orchratoxin A production by two new species, *Aspergillus aculeatinus* and *A. sclerotiicarbonarius*, on a green coffee-based medium. International Journal of Food Microbiology. 2014;**188**:116-121. DOI: 10.1016/j.ijfoodmicro.2014.07.025

[13] Fleurat-Lessard F. Integrated management of the risks of stored grain spoilage by seedborne fungi and contamination by storage mould mycotoxins—An update. Journal of Stored Products Research. 2017;**71**:22-40. DOI: 10.1016//j.jspr.2016.10.002

[14] Mohapatrab D, Kumara S, Kotwaliwaleb N, Singha KK. Critical factors responsible for fungi growth in stored food grains and non chemical approaches for their control. Industrial Crops and Products. 2017;**108**:162-182. DOI: 10.1016/j.indcrop.2017.06.039

[15] Abrunhosa L, Morales H, Soares C, Calado T, Vila-Chä AS, Pereira M, et al. Micotoxinas detectadas en productos alimenticios en Portugal: Revision. Revista Bio Ciencias. 2012;**3**:5-31

[16] Singh P, Shukla R, Prakash B, Kumar A, Singh S, Kumar MP, et al. Chemical profile, antifungal, antiaflatoxigenic and antioxidant activity of *Citrus maxima* Burm. and *Citrus sinensis* (L.) osbeck essential oils and their cyclic monoterpene, dl-limonene. Food and Chemical Toxicology. 2010;**48**:1734-1740. DOI: 10.1016/j.fct.2010.04.001

[17] Neme K, Mohammed A. Mycotoxin occurrence in grains and the role of postharvest management as a mitigation strategies. A review. Food Control. 2017;**78**:412-425. DOI: 10.1016/j.foodcont.2017.03.012

[18] Malir F, Ostry V, Grosse Y, Roubal T, Skarkova J, Ruprich J. Monitoring the mycotoxins in food and their biomarkers in the Czech Republic. Molecular Nutrition & Food Research. 2006;**50**:513-518. DOI: 10.1002/mnfr.200500175

[19] Kensler TW, Roebuck BD, Wogan GN, Groopman JD. Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. Toxicological Sciences. 2011;**120**:S28-S48. DOI: 10.1093/toxsci/kfq283

[20] Ostry V, Frantisek M, Jakub T, Yann G. Mycotoxins as human carcinogens—The IARC monographs classification. Mycotoxin Research. 2017;**33**:65-73. DOI: 10.1007/s12550-016-0265-7

[21] IARC. Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. Vol. 56. Lyon, France: International Agency for Research on Cancer; 1993. pp. 1-599. Available from: https://monographs.iarc.fr/wp-content/uploads/2018/06/mono56.pdf. [Accessed: November 22, 2018]

[22] IARC. Preamble to the IARC Monographs. Lyon, France: International Agency for Research on Cancer. 2006. Available from: https://monographs.iarc.fr/preamble-to-the-iarc-monographs-amended-january-2006/ [Accessed: November 23, 2018]

[23] IARC. Presentations Related to the IARC Monographs. IARC Monographs, Evaluations; IARC, Lyon. 2016. Available from: http://monographs.iarc.fr/ENG/Publications/Evaluations.pdf [Accessed: November 23, 2018]

[24] IARC. Agents Classified by the IARC Monographs, Vol. 1-116. 2016. Available from: http://monographs.iarc.fr/ENG/Classification/ [Accessed: November 24, 2018]

[25] Richard J. Some major mycotoxins and their mycotoxicoses an overview. International Journal of Food Microbiology. 2007;**119**:3-10. DOI: 10.1016/j.ijfoodmicro.2007.07.019

[26] Koblitz BGM. Matérias Primas Alimentícias. Composição e Controle de Qualidade. Rio de Janeiro: Ed Guanabara Koogan; 2011

[27] World Health Organization. Food Safe Digest. Aflatoxins. Ref. No.: WHO/NHM/FOS/RAM/18.1. 2018. Available from: https://www.who.int/foodsafety/FSDigest_Aflatoxins_EN.pdf [Accessed: November 26, 2018]

[28] Ribeiro CLN, Barreto SLT, Hannas MI. Micotoxinas encontradas em rações e alimentos utilizados na produção comercial de aves no Brasil. Revista Eletrônica Nutritime. 2015;**12**:3910-3924. Available from: http://www.nutritime.com.br/arquivos_internos/artigos/ARTIGO292.pdf

[29] Freire FCO, Vieira ÍGP, Guedes MIF, Mendes Pereira NF. Micotoxinas: Importância na alimentação e na saúde Humana e Animal Fortaleza; Embrapa Agroindústria Tropical. 2007. p. 48. Available from: https://ainfo.cnptia.embrapa.br/digital/bitstream/item/30348/1/Marcelo-A-Braz.-J.-Food-Technol.pdf [Accessed: December 10, 2018]

[30] Channaiah L. Mycotoxin. World-Grain.com. 2011. Available from: https://www.world-grain.com/News/News%20Home/Features/2011/6/Mycotoxins.aspx?cck

[31] Adeyeye OAS. Fungal mycotoxins in foods: A review. Cogent Food & Agriculture. 2016;**2**:1213127. DOI: 10.1080/23311932.2016.1213127

[32] Pfohl-Leszkowicz A, Manderville RA. Ochratoxin A: An overview on toxicity andcarcinogenicity in animals and humans. Molecular Nutrition & Food Research. 2007;**51**:61-99. DOI: 10.1002/mnfr.200600137

[33] Jakub T, Ostry V, Grosse Y, Roubal T, Malir F. Occurrence of ochratoxin A in Astragalus propinquus root and its transfer to decoction. Mycotoxin Research. 2018;**34**:223-227. DOI: 10.1007/s12550-018-0317-2

[34] Marín S, Mateo ME, Sanchis V, Valle-Algarra MF, Ramos JA, Jiménez M. Patulin contamination in fruit derivatives, including baby food, from the Spanish market. Food Chemistry. 2011;**124**:563-568. DOI: 10.1016/j.foodchem.2010.06.072

[35] Qileng A, Wei J, Lu N, Liu W, Cai Y, Chen M, et al. Broad-specificity photoelectrochemical immunoassay for the simultaneous detection of ochratoxin A, ochratoxin B and ochratoxin C. Biosensors and Bioelectronics. 2018;**106**(30):219-226. DOI: 10.1016/j.bios.2018.02.004

[36] Iqbal ZS, Malik S, Asi RM, Selamat J, Malik N. Natural occurrence of patulin in different fruits, juices and smoothies and evaluation of dietary intake in Punjab, Pakistan. Food Control. 2018;**84**:370-374. DOI: 10.1016/j.foodcont.2017.08.024

[37] Pestka JJ. Deoxynivalenol: Mechanisms of action, human exposure, and toxicological relevance. Archives of Toxicology. 2010;**84**:663-679. DOI: 10.1007/s00204-010-0579-8

[38] Piacentini CK, Rocha OL, Fontes CL, Carnielli L, Reis AT, Corrêa B. Mycotoxin analysis of industrial beers from Brazil: The influence of fumonisin B1 and deoxynivalenol in beer quality. Food Chemistry. 2017;**218**:64-69. DOI: 10.1016/j.foodchem.2016.09.062

[39] Sun J, Wu Y. Evaluation of dietary exposure to deoxynivalenol (DON) and its derivatives from cereals in China. Food Control. 2016;**69**:90-99. DOI: 10.1016/j.foodcont.2016.04.040

[40] Hueza MI, Raspantini FCP, Raspantini REL, Latorre OA, Górniak LS. Zearalenone, an estrogenic mycotoxin, is an immunotoxic compound. Toxins. 2014;**6**:1080-1095

[41] Metzler M, Pfeiffer E, Hildebrand AA. Zearalenone and its metabolites as endocrine disrupting chemicals. World Mycotoxin Journal. 2010;**3**(4):385-401

[42] Kowalska K, Górczyńska H, Ewa D, Domińska K, Piastowska-Ciesielska AW. The dose-dependent effect of zearalenone on mitochondrial metabolism, plasma membrane permeabilization and cell cycle in human prostate cancer cell lines. Chemosphere. 2017;**180**:455-466. DOI: 10.1016/j.chemosphere.2017.04.027

[43] Domijan A-M. Fumonisin B1: A neurotoxic mycotoxin. A review. Archives of Industrial Hygiene and Toxicology. 2012;**63**:531-544. DOI: 10.2478/10004-1254-63-2012-2239

[44] Federico JF, Funes JG, Salas PM, Resnik LS. Natural occurrence of fumonisins in corn puff in Argentina. Mycotoxicology Research. 2010;**26**:273-278. DOI: 10.1007/s12550-010-0066-3

[45] Freire L, Sant'Ana SA. Review: Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects. Food and Chemical Toxicology. 2018;**111**:189-205. DOI: 10.1016/j.fct.2017.11.021

[46] Humpf UH, Rychlik M, Cramer B. Modified mycotoxins: A new challenge? Reference module in food science. In: Encyclopedia of Food Chemistry. Germany: Elsevier; 2019. pp. 393-400. DOI: 10.1016/B978-0-08-100596-5.21802-7

[47] Czabana J, Wróblewsk B, Sułek A, Mikos M, Boguszewska E, Podolska G, et al. Colonisation of winter wheat grain by *Fusarium* spp. and mycotoxin content as dependent on a wheat variety, crop rotation, a crop management system and weather conditions. Food Additives & Contaminants: Part A. 2015;**32**(6):874-910. DOI: 10.1080/19440049.2015.1019939

[48] Calado T, Fernández-Cruz LM, Cabo-Verde S, Venâncio A, Abrunhosa L. Gamma irradiation effects on ochratoxin A: Degradation, cytotoxicity and application in food. Food Chemistry. 2018;**240**:463-471. DOI: 10.1016/j.foodchem.2017.07.136

[49] Zinedine A, Soriano MJ, Moltó CJ, Mañes J. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. Food and Chemical Toxicology. 2007;**45**(1):1-18. DOI: 10.1016/j.fct.2006.07.030

Chapter 4

Cell Growth Measurement

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Abstract

The cell is the basic structural and functional unit of all living organisms. As the smallest unit and building blocks of life, cells differ in size, shape, metabolism, reproduction, and growth requirements. Cells reproduce through cell division involving a four-phase (G1, S, G2, M) cell cycle, which is tightly regulated at multiple checkpoints. The resulting growth curve demonstrates that cell population increases in three sequential steps: incubation, exponential hyperplasia, and stagnation/death phases. Cell growth is subject to changes in disease state and/or environmental conditions. This chapter will focus on methods for cell growth measurement, which are grouped into five sections: cell cycle, apoptosis, growth curve, drug-induced proliferation (DIP), and continuous assays. Among the continuous assays, the EZMTT dye allows for long-term tracking of cell growth under various conditions and shows promise in precision medicine by early detection of drug resistance.

Keywords: cell cycle, apoptosis, growth curve, drug-induced proliferation, continuous assays, drug resistance

1. Introduction

The cell is the smallest unit of living organisms [1] and grows both in population and size. Cellular growth [2] is tightly regulated and usually shows three sequential steps, including incubation, exponential hyperplasia, and stagnation/death phases [3]. Unrestricted cell growth causes cancer, and drugs cure the disease by regulating the cell growth back to normal. Therefore, precise measurement of cell growth is very important in biomedicine, including cancer, aging, drug resistance, drug discovery, environment contamination, material biocompatibility [4, 5], fermentation, immunology, etc.

Cells grow by cell division which includes four major components: the G1, S, G2, and M phases in sequence [6], as shown in **Figure 1**. The signature of the G1 phase is the synthesis of enzymes that are required for DNA replication. During the S phase, DNA is replicated to produce two identical sets of chromosomes. The G2 phase is mainly involved in the production of microtubules that are required during the process of division, the mitotic phase. Increases in cell volume are observed during the interphase (G1, S, G2 phase). The M phase consists of prophase, metaphase, anaphase, and telophase in sequence, and the parent cell is divided into two daughter cells through nuclear division (karyokinesis), cytoplasmic division (cytokinesis), and formation of a new cell membrane [7].

Cell division is more complex in eukaryotes whose cell division involves either mitosis or a more complex process called meiosis. Mitosis and meiosis are two

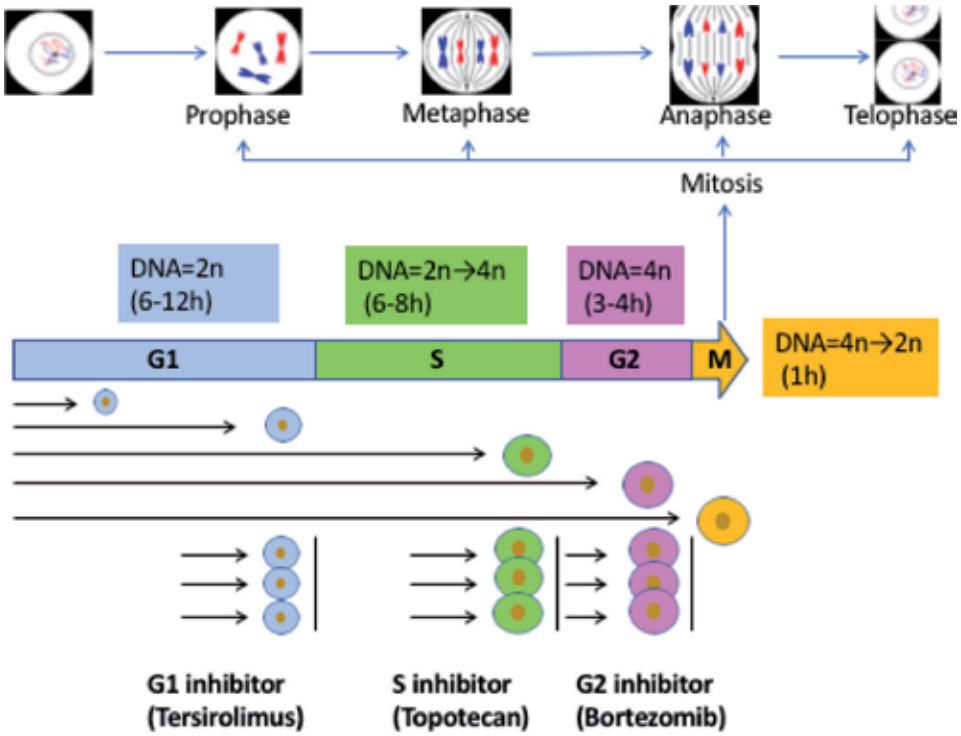

Figure 1.
Cell cycle.

different "nuclear division" processes. Through binary fission, mitosis [8] produces two daughter cells with the same number of chromosomes as the parental cell. Meiosis, also called reductive division, is the division of a germ cell involving two fissions of the nucleus to form four gametes that have half the normal cellular amount of DNA. A male and a female gamete can then combine to produce a zygote, a cell which again has the normal number of chromosomes [9]. Therefore, the enlargement of cell volume and changes in DNA content are two parameters commonly used in cell cycle measurement.

The cell cycle is tightly regulated at multiple checkpoints [10]. Various growth conditions such as the temperature, nutrients, cell density, and drug treatment can block the cell cycle at various stages. Instead of unrestricted growth, the cell growth curve shows that the cell population increases through three phases: incubation period → exponential hyperplasia → stagnation period. During exponential growth, cells demonstrate great variation in required cell density and doubling times that are highly dependent on cell type and growth conditions.

Cell death occurs in each generation. Acute cellular injury causes traumatic cell death (necrosis) [11], whereas apoptosis is a highly regulated and programmed cell death that occurs each day in multicellular organisms. The average adult human loses between 50 and 70 billion cells each day due to apoptosis [12] which is critical, because uncontrolled cell proliferation is closely related to the occurrence of human diseases such as tumors. Commonly used analyses for apoptosis are morphological analysis, detection of apoptotic biomarkers, and flow cytometric analysis of cellular DNA content.

Inhibiting cancer cell or infectious microbial growth is the purpose of drug treatment. However, drug resistance is the notorious worldwide crisis that prolongs hospital stays and considerably increases mortality. Identification of specific genetic mutations has been the major effort in understanding drug resistance, but the

results have had little diagnostic value [13]. Recently, many research groups [14–17] demonstrated that drug resistance develops owing to a small population of cells resistant to the drug, and drug treatment results in the selection for the growth of the small drug-resistant cell population. Recognition of partial efficacy is, therefore, very important in early detection of drug resistance.

Traditionally, the dose–response curve is used to evaluate the potency of an inhibitor (IC_{50}). Commonly used cell proliferation assays are metabolic activity-based methods such as the tetrazo-based cellular NAD(P)H detection system (MTT, CCK8, EZMTT) and the cellular ATP detection system (CellTiter-Glo Assay). Unfortunately, due to experimental error, the endpoint assays are not sensitive enough to detect the survival of a minor population of cells.

For precise measurement of drug efficacy, the drug-induced proliferation (DIP) rate has been proposed as a better parameter than the IC_{50} or MIC (80% inhibition) measurement [15]. However, the measurement of a precise DIP rate calls for continuous assays that can be used easily with various cell types.

Therefore, this chapter will mainly discuss the methods used for measuring cell cycle, apoptosis, growth curve, and drug-induced proliferation, continuous assays that can track the growth condition-induced cell proliferation, and its applications in early discovery of drug resistance.

2. Cell cycle

Each cell cycle involves the G1, S, G2, and M phases in sequence, and each phase is associated with its signature protein biomarkers, DNA content, and cell size, as shown in **Figure 1**. DNA ploidy and protein biomarker analyses are commonly used in cell cycle studies and have important applications in clinical cancer diagnosis, drug efficacy evaluation, prognosis prediction, cell dynamics, and apoptosis.

2.1 Parameters used for DNA content analysis

DNA ploidy, DNA index (DI), S phase fraction (SPF), and potential doubling time (Tpot) are commonly used parameters for cell cycle analyses.

DNA ploidy refers to the number of chromosomes or the total DNA content in cells. DNA ploidy analysis in combination with clinical pathological diagnosis has a great value in early diagnosis and in prognosis prediction for malignant tumors, for both solid tumors and cancer cell extracts from body fluids, glandular secretions, and exfoliated tissue cells. Aneuploid tumors showed significantly higher recurrent rates than the diploid ones [18].

The DNA index (DI) refers to the ratio between the cells in G0/G1 peak of the tumor samples and that of the normal diploid samples. The calculation equation is DI (DNA index, number of judgment ploidy) = (average number of G0/G1 phase cell peaks in sample)/(average number of G0/G1 phase cell peaks in normal diploid cells). A DI of 1 means a normal diploid sample (generally the normal range is 0.9 to 1.1) [19].

The S phase fraction (SPF) shows the percentage of cells in S phase and indicates the proliferative activity of the cells. The calculation equation is SPF (S phase fraction %) = S cells / (G0/G1 + S + G2M) cells × 100%. Another cell proliferation parameter is proliferation index (PI), and the calculation equation is PI (proliferation index %) = (S + G2M) cells/ (G0/G1 + S + G2M) cells × 100%.

The potential doubling time (Tpot) refers to the time required to double the cell number, which occurs during exponential growth.

2.2 DNA content analysis

DNA content can be analyzed after fluorescent staining or labeled nucleic acid incorporation as shown in **Table 1**.

As shown in **Figure 2**, DAPI and Hoechst dyes penetrate the membrane and are commonly used to label live cells, whereas propidium iodide (PI), propidium monoazide (PMA), and ethidium bromide monoazide (EMA) only label dead cells. Newly synthesized DNA in active proliferating cells can be labeled by the radiolabeled ^{3}HTdR, ^{125}IUdR [24], or the fluorescent-labeled BrdU and EDU [25].

Flow cytometry measurement (FCM) is a sensitive method to measure cell size and fluorescent labeling. Double staining both cellular DNA and protein biomarker allows identification of cells in G1 peaks, G2+ M peaks, and S platforms, as well as the subdiploid peaks (apoptotic peaks) before the G1 peak. These methods in combination with other biomarkers show the distribution of cells in each phase of the cell cycle and can be used to investigate cell dynamics [26].

2.3 Protein biomarkers

Proteins that are found in proliferating cells, but not in nonproliferating cells can be used as biomarkers for cell cycle measurement. Ki-67 protein (also known as MKI67) is present during all active phases of the cell cycle (G1, S, G2, and mitosis) but is absent in resting (quiescent) cells (G0) [27]. During interphase, the Ki-67 protein is exclusively located in the cell nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. During cell progression through S phase of the cell cycle, the Ki-67 protein markedly increases [28]. As shown in **Figure 1**, the fluorescent-labeled monoclonal Ki-67 antibody has been used for cell cycle measurement and cancer diagnosis. Other commonly used cell proliferation biomarkers include proliferating cell nuclear antigen PCNA [29], topoisomerase IIB [30], and phosphorylated histone H3 [31].

DNA stain	Principle	Membrane permeability	Function
4′,6-diamidino-2-phenylindole (DAPI)	Binds to DNA A-T rich region [20]	Yes	Live cell
Hoechst dye	Binds to DNA A-T rich region [21]	Yes	Live cell
Propidium Iodide (PI)	Label dead cells [22]	No	Dead cell
Propylene glycol monomethyl ether acetate (PGMEA)	In combination with dsDNA to form a stable and strong covalent nitrogen-carbon bond [23]	No	Dead cell
Ethidium bromide monoazide (EMA)	Covalent cross-linking with genomic DNA [23]	No	Dead cell
3 HTdR incorporation	^{3}H-TdR incorporates in DNA synthesis	Yes	Live cell
125IUdR incorporation	^{125}IUdR incorporates in DNA synthesis	Yes	Live cell
BrdU incorporation	Brdu participates in DNA synthesis in cell proliferation	Yes	Live cell
EDU incorporation	EDU participates in DNA synthesis in cell proliferation	Yes	Live cell

Table 1.
Comparison of methods for DNA labeling.

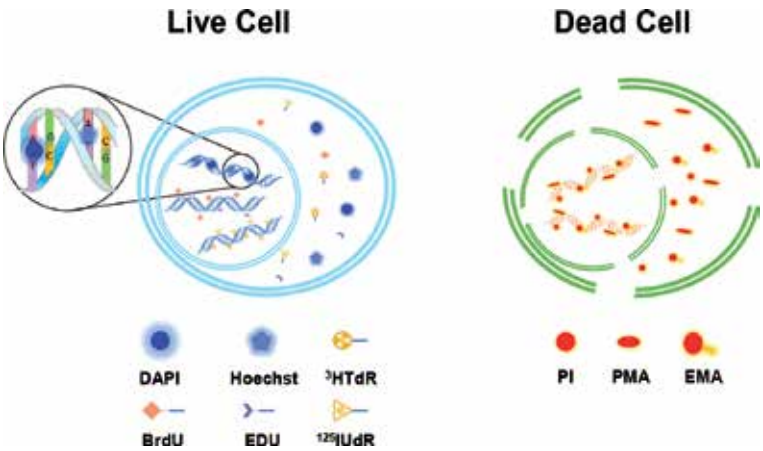

Figure 2.
Methods for DNA analysis in both live and dead cells.

3. Apoptosis

Apoptosis is a programmed cell death, and the process involves a series of morphological changes such as blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, as well as biochemical changes such as chromosomal DNA fragmentation, global mRNA decay [32], and appearance of protein biomarkers in protein degradation pathways.

Commonly used analyses include transmission electron microscopy for morphological analysis, biochemical assays for detection of apoptotic biomarkers, and flow cytometry analysis of cellular DNA content (**Figure 3**).

3.1 Transmission electron microscopic analysis

Tissue or cells can be directly stained using dyes such as hematoxylin, methyl green pyronine, and acridine orange for microscopic analysis. Under the transmission electron microscope [33], apoptotic cells show reduced size and more concentrated cytoplasm. In the nucleus of pro-apoptosis phase, the chromatin is highly coiled, and many vacuole structures called cavitation appear; in the phase IIa nucleus, the chromatin is highly coagulated and marginalized; in the end, the nucleus is cleaved into fragments and produces apoptotic bodies. Fluorescence microscopy and confocal laser scanning microscopy [34] can also be used to observe the progress of apoptosis, based on morphological changes of nuclear chromatin DNA-specific dyes such as Hoechst dye series and DAPI.

3.2 TUNEL method

The TUNEL assay [35] distinguishes between normal and apoptotic cells based on the amount of FITC-dUTP incorporation into the broken DNA ends in the intact single apoptotic nuclei or apoptotic bodies, whereas the normal or growing cells have almost no DNA breaks and will not be stained. This method accurately reflects the most typical biochemical and morphological features of apoptosis and can used with paraffin-embedded tissue sections, frozen tissue sections, cultured cells, and tissue-separated cells. The method is simple, easy, and sensitive in detecting a very small amount of apoptotic cells.

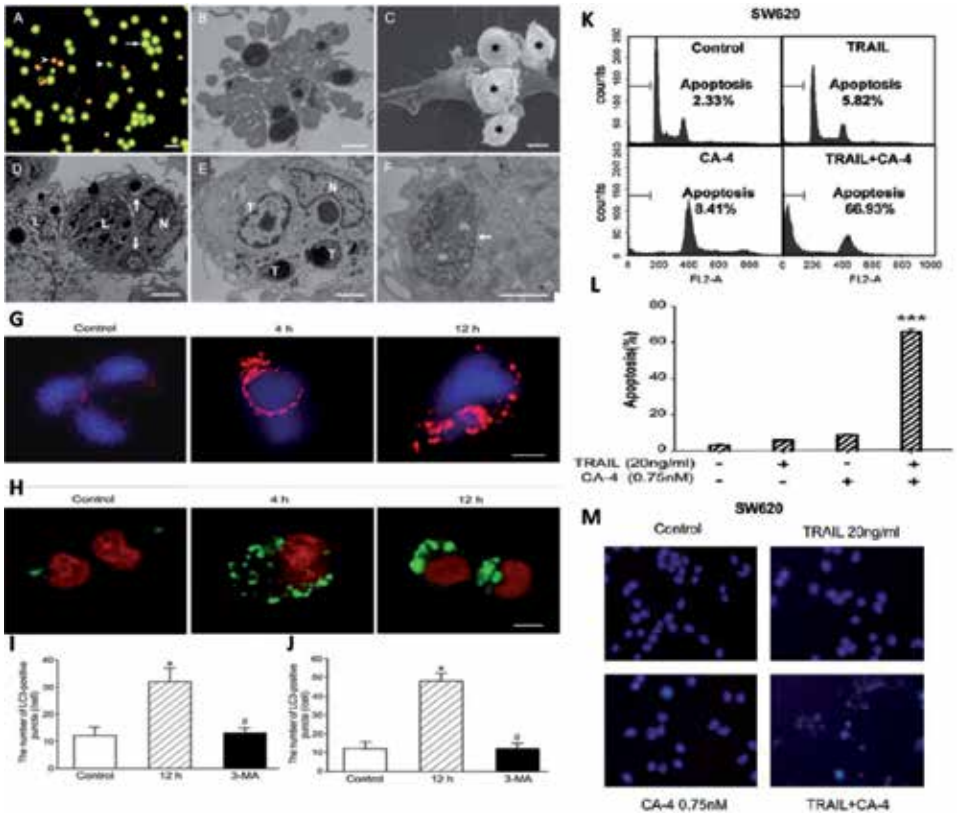

Figure 3.
Methods in measuring apoptosis: (A) lymphocytes stained with EB/AO solution. Triangle, arrow, and arrowhead show viable, early apoptotic and late apoptotic cells, respectively. (B) Transmission electron image of a broken apoptotic lymphocyte. The cell breaks up into apoptotic bodies containing organelles or the condensed nuclear fragments. (C) Scanning electron image of a macrophage phagocytosing the apoptotic lymphocytes (asterisks). (D) A macrophage engulfing an apoptotic lymphocyte L, containing the condensed nuclear fragments. Arrows show phagosomes containing the apoptotic bodies of lymphocytes. (E, F) A macrophage phagocytosing apoptotic cells. (G–J) LC3 fluorescent stain in macrophages phagocytosing the apoptotic lymphocytes. The nuclei were stained with DAPI (G) or PI (H). (K and L) PI staining was used to evaluate the apoptosis.

The limitations of the TUNEL method are as follows. Firstly, it labels apoptotic cells only in the middle and late stages. Secondly, necrotic cells also contain DNA breaks and are labeled by dUTP, so the assay is not specific enough to distinguish between apoptotic and necrotic cells. Thirdly, cells need to be fixed during the labeling process, which may lead to excessive cell debris or loss of DNA fragments. Lastly, subjective factors are involved when counting the number of the apoptotic cells.

In addition, agarose gel electrophoresis can be used to detect the apoptotic DNA fragmentation into an integer multiple of 180–200 bp [36]. ELISA can be performed using anti-DNA and anti-histone monoclonal antibody to detect nucleosome fragments [37]. Mitochondrial membrane potential measurement can distinguish early apoptosis [38] and provides a nice complement to the TUNNEL method.

3.3 Fluorescent staining for flow cytometry analysis

Application of annexin V is a widely used method for detecting apoptosis [39]. Annexin V is a Ca2 + −dependent phospholipid binding protein with a molecular weight of 35–36KD, which can bind with phosphatidylserine with high

affinity. Annexin V can be labeled with fluorescein isothiocyanate (FITC, PE) or biotin and used as a fluorescent probe to detect apoptosis by flow cytometry or fluorescence microscopy.

PI dye does not pass through the intact cell membrane, but in the middle and late stages of apoptosis, PI can pass through the cell membrane to bind to the DNA and redden the nucleus [40, 41]. PI staining of the apoptotic cells shows a subdiploid peak (Ap peak) that appears before the G1 peak. The number of apoptotic cells can be detected based on the level of the Ap peak. Therefore, combining results from both annexin V staining and PI staining, cells in the early and late stage of apoptosis and necrosis can be distinguished.

However, the limitation of both staining methods is the poor sensitivity. It is difficult to detect DNA fragments in early apoptotic cells and easy to miss the detection of apoptotic cells in S phase or G2/M phase. Meanwhile, the PI method is partially necrotic, and the cellular debris could cause false detection.

Additional methods are detecting the release cytochrome C from mitochondria into cytoplasm during apoptosis [42], as well as activation of signaling pathways involving caspase 3, caspase 9, Apaf-1, PARP, Bcl2, Akt, TFAR19, etc., to differentiate the exogenous or endogenous cause of cell apoptosis [43].

3.4 Double or multiple staining

To distinguish the apoptotic cells, necrotic cells, and living cells, researchers perform flow cytometric analysis after double staining with annexin V and PI. Annexin V-FITC has poor membrane permeability and can specifically bind phosphatidylserine; for live cells, no phosphatidylserine can be detected by annexin V, but during apoptosis, phosphatidylserine is valgus outward to the outer side of the cell membrane for easy detection. The fluorescent dye PI binds to chromatin but does not enter the cytoplasm of live cells. However, PI can enter the apoptotic cells and necrotic cells and effectively stain the concentrated chromatin in apoptotic cells. In addition, for low PI-stained cells, H0342 dye can be used for detection of apoptotic cells with concentrated chromatin. H0342 stain enters cells with intact membranes and stains apoptotic cells more strongly than normal cells. When used in combination with forward scatter (FSC) and side scatter (SSC), H0342 staining can distinguish apoptosis from living cells [44].

Taken together, the FCM method requires less sample, provides high sensitivity, and simultaneously analyzes apoptotic cells and normal cells. Morphological observation can be carried out using an optical microscope, and electron microscopy is an authoritative method, which is commonly used before serious quantitative analysis. Agarose gel electrophoresis can also be used as a qualitative analysis, but the results need further confirmation by TUNNEL technology or annexin-V/PI dual-label method.

4. Cell proliferation

Cell growth (proliferation) can be evaluated by the time-dependent changes of the total number of proliferating cells, as well as the ratio of cells among individual phases of the cell cycle. The cell growth curve was plotted to show the time-dependent increase in cell numbers. Depending on the cell type and growth condition, the required cell density to enter the exponential phase and the rate of cell growth measured by the doubling time (Tpot) could be very different. For example, most microbials (e.g., *E. coli*) enter the exponential growth phase with low cell numbers and show fast growth rate with a doubling time less than an hour [45],

whereas *M. tuberculosis* and mammalian cells have longer doubling time of several hours or a day. The growth and reproduction of cells can be further altered by changes in temperature, nutrition, viral infection, and/or the presence or absence of inhibitors [46]. Therefore, the growth curve is important in guiding clinical drug usage, investigating gene functions, and understanding drug mechanism of action. Various methods have been developed to measure the absolute number of cells or the changes in cell number, as shown in **Figure 4**.

4.1 Manual cell counting

Traditionally, cell numbers are counted by taking an aliquot of a homogenous cell suspension and plating on a hemocytometer to count the numbers under a light microscope. The obtained cell number in a certain volume of the suspension is then converted into the cell concentration (cells per ml) in the stock solution. Bacteria are counted by a Petroff-Hausser bacterial counter, a Hawksley counter, and/or the plate colony formation method. The plate colony counting method often gives a lower cell number than the actual value, because it is often difficult to disperse bacteria into a single cell and to make sure that a single colony is not derived from several bacteria.

4.2 Automated mechanical counting

The most commonly used automatic cell counting methods are direct electrical impedance, flow cytometry, computer-aided image analysis, and serological counting. Through changes in electrical properties, the direct electrical impedance method quantifies the number and the volume of cells in the blood. Using a photomultiplier to filter and detect the signal, flow cytometry records both the density and height of fluorescent pulses and then converts them to the number of bacteria; the method is fast and sensitive and can simultaneously analyze the cell morphology and protein biomarkers. Computer-aided image analysis [47] and serology [48] counting methods analyze the image or 2D picture to obtain accurate quantification and morphological structure. So far, both methods have been used successfully in biology, materials science, mineralogy, and neurological science.

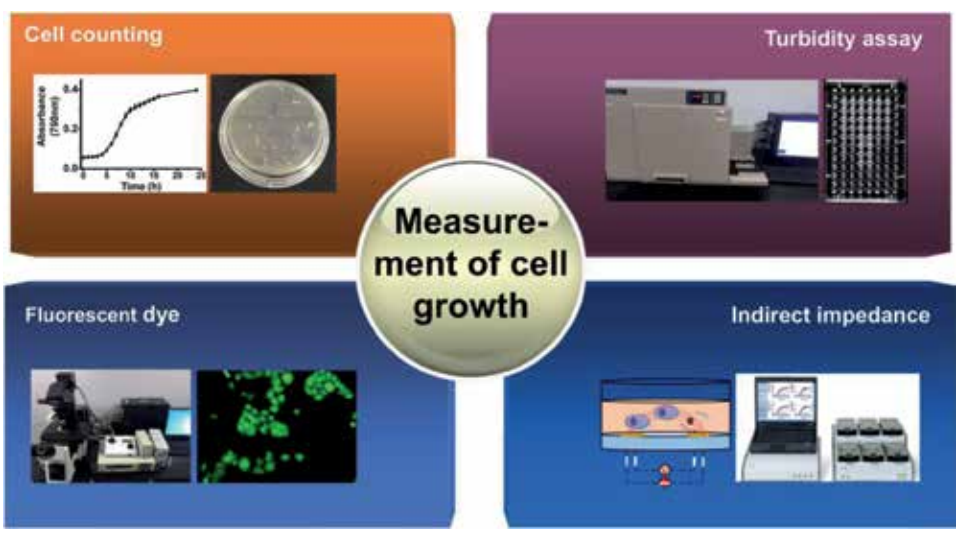

Figure 4.
The main methods for cell growth measurement.

4.3 Indirect cell counting methods

4.3.1 Turbidity assay by spectrophotometry

Turbidity can be observed when the cell density reaches certain level; within a certain range, the number of cells is proportional to the turbidity of the bacterial culture. The cell turbidity is measured by a spectrophotometer or a colorimeter, and a standard curve is generated by plotting the absorbance at OD600nm and the actual cell numbers in the sample. Photoelectric turbidimetric counting is a simple, rapid, and continuous measurement suitable for high-throughput screening. However, its optical density is less sensitive, cannot differentiate between dead or live bacteria, and is greatly affected by cell size, morphology, and the color of the culture solution [49].

4.3.2 BACTEC MGIT method

BACTEC MGIT [50, 51] measures microbial growth by oxygen depletion which requires anaerobic conditions, so the bacteria must be grown in a sealed tube or compartment. This method has been widely applied in medical diagnosis but is not suitable for high-throughput plate-based AST assays.

4.3.3 Fluorescent dye method

Live or dead cells that cannot be differentiated by the light microscope can be counted after fluorescent labeling. **Table 2** showed the commonly used dyes that

Fluorescent dye	Stain subject	Membrane permeability	Excitation/ emission wavelength	Function	Detection
SYTO nucleic acid stains	Nucleic acid [52]	Yes	420~657/441~678	Live or dead cell	Fungus, bacteria
SYTOX green nucleic acid stain	Nucleic acid [53]	No	~504/~523	Dead cell	Fungus, bacteria
Propidium iodide (PI)	Nucleic acid [54]	No	~530/~635	Dead cell	Fungus, bacteria, mammals
Sulforhodamine B (SRB)	Protein [55]	Yes	~565/~586	Live cell	Mammals
PHK26, 67	Membrane [56]	Yes	~551/~567, ~496/~520	Live cell	Mammals
DiO	Membrane [57]	Yes	482~487/ 501~504	Dead cell	Mammals
DiD	Membrane [58]	Yes	~646/~665	Dead cell	Mammals
Calcofluor white M2R	Cytoderm [59]	Yes	385~405/ 437~445	Live cell	Fungus
DiBAC4(3)	Membrane [60]	Yes	506/ 526	Dead cell	Fungus, bacteria, mammals

Table 2.
Summary of fluorescent dye.

label the cellular components such as nucleic acid, cytoplasm, cell membrane, redox environment, and lipase. For example, SYTO series nucleic acid fluorescent dyes, etc. stain the DNA or RNA of the live or dead cells; PI nor SYTOX Green nucleic acid dyes cannot transfer into the live cells and stain the DNA of the damaged cell membranes. Both types of dyes can be used in combination to measure the ratio of live and dead cells. After labeling, the cells can be detected by a fluorescence microscopy or by a flow cytometry. The combination of fluorescent dyes and advanced instruments makes it possible to realize the "visualization" and investigate the mechanisms of action under the physiological and pathological conditions and to explain the significance of life effects, which is of great significance in the field of disease diagnosis and drug screening.

5. Drug-induced proliferation assays

A drug is a substance that induces functional changes in an organism through chemical or physical actions, regardless of whether the resulting effect is beneficial or detrimental to the health of the receiving organism. Therefore, assays are critical in revealing interactions between the drug and the organism. In particular, the cell proliferation assays provide valuable information for exploring the pathogenesis of the disease, diagnosing the disease, and treating the disease.

Traditionally, a dose–response curve is used to evaluate the potency of an inhibitor (IC_{50}). The commonly used cell proliferation assays are either metabolic activity-based methods such as the tetrazo-based cellular NAD(P)H detection system (MTT, CCK8 method) or the cellular ATP detection system (CellTiter-Glo Assay). According to the mechanism of metabolic activity detection, these endpoint colorimetric and fluorescence methods can be divided into five categories (**Table 3**): the reducing environment of live cells, ATP of live cells, detecting products released by dead cells, esterase, and detecting mitochondrial metabolism of live cells.

5.1 Reducing environment of live cells

Resazurin and its derivative C12-resazurin are not fluorescent, but their reduced forms are fluorescent. Dehydrogenase reduces the non-fluorescent blue resazurin into a strongly fluorescent pink resorufin [61] in enzyme- or cell-based assays. Since the dehydrogenase level is high in active cells and very low in damaged or inactive cells, the resazurin assay shows a strong signal in metabolically active cells. Interestingly, even though resazurin can be reduced by mitochondrial enzymes, no evidence of resazurin reduction was found in mitochondria as shown by confocal microscopy analysis [62].

Resazurin is water-soluble and stable in culture medium. Single reagent addition allowed for simple assays of cell viability which is especially suitable for automated manipulation and high-throughput analysis [63]. However, the fluorescence is bleached by light, so it is not suitable to track the cell growth.

5.2 ATP production in live cells

Adenosine triphosphate (ATP) is an indicator of active live cells, and its cellular level directly reflects the number and state of cells. The CellTiter-Glo method is a luminescence-based endpoint assay, commonly used for ATP measurement after cell lysis. The assay is based on ATP consumption by luciferase to produce light (maximum emission wavelength ~560 nm at pH 7.8) [64]. Due to the absence of interference of endogenous luciferase in mammalian cells, a stable glow-type signal

Classification	Method	Description	Cell viability	Application
Reducing environment of live cells	Resazurin	Reduced by cytosolic dehydrogenase in metabolically active cells to produce pink strong fluorescent resorufin	Live cells	Growth
ATP production in live cells	CellTiter-Glo	Luciferase consumes ATP to produce light	Lysed cells	Growth
Esterase of live cells	FDA;calcein-AM	Cleaved by esterase in living cells to produce a fluorescent substance retained in the cell	Live cells	Division
Released products of dead cells	LDH release	LDH released by damaged or dead cells reduces the tetrazolium salts to colored formazan	Dead cells	Death
	^{51}Cr release	51Cr released by damaged or dead cells can be measured by a radioactivity assay	Dead cells	Death
Mitochondrial dehydrogenase of live cells	Tetrazolium	Reduced by mitochondrial dehydrogenase to produce colored formazan products	Live cells	Growth

Table 3.
Comparison of commonly used methods for drug-induced proliferation assays.

generated using luciferase can be detected as little as 0.1 picomoles ATP by luminometers, showing high sensitivity, although the signal decreases after 10–30 min.

The luciferin-luciferase bioluminescence assay has been used to detect small amount of bacterial contamination in samples such as blood, milk, urine, soil, and sludge [65]. In addition, the assay can evaluate antibiotic effects, determine cell proliferation and cytotoxicity in both bacterial and mammalian cells [64], and distinguish the cytostatic and killing potential of anticancer drugs for malignant cell growth. Furthermore, it has been used for bioactive factor activity assays, large-scale antitumor drug screening, cytotoxicity assays, and tumor radiosensitivity assays.

5.3 Lactonase in living cells

Lactonase is a non-specific esterase and cleaves a non-fluorescent molecular probe to produce a fluorescent substance. Lactonase activity is high in live cells but low in dead cells, so the fluorescence is seen and retained in live cells. In addition, the fluorescence can be evenly distributed to the two daughter cells after cell division, although each successive passage decreased the fluorescence intensity in cells by 50% as analyzed by flow cytometry [66].

The most commonly used fluorescent probes are fluorescein diacetate (FDA) and its derivative succinimidyl ester of carboxyfluorescein diacetate, commonly known as CFSE-SE [67]. CFDA-SE was initially used for lymphocyte proliferation testing in 1994 and then applied to detect monocytes, fibroblasts, etc. The CFDA-SE method determines the cycle number of cell divisions based on fluorescence intensity. Also, it can be used to trace in vivo studies [68, 69].

In addition, calcein-AM, (3′,6′-Di(O-acetyl)-4′,5′-bis[N,N-bis(carboxymethyl) aminomethyl] fluorescin tetraacetoxymethyl ester), also known as calcein acetyl

ester, is a fat-soluble substance and membrane-permeable fluorescein dye [70]. It is non-fluorescent and freely enters the cytoplasm and organelles such as the mitochondrial matrix. Upon entry, calcein-AM can be hydrolyzed by intracellular esterase to produce water-soluble calcein (calcium chlorophyll), which remains in the cytosol and mitochondrial matrix and produces strong green fluorescence under 494 mm excitation light.

5.4 Released products from dead cells

The radioisotope chromium (51Cr) release method was initially developed to detect dead cells and has now gradually been replaced by the LDH release method. Due to increased membrane permeability, the damaged or dying cells release the cytosolic LDH in cell culture medium [71], so the LDH activity is proportional to the number of dead cells. The LDH release assay has been used to measure the activity of cytotoxic lymphocyte (CTL) and natural killer (NK) cells, as well as the cytotoxicity caused by drugs, chemicals, or radiation.

5.5 NADH and NADPH production of live cell

NADH and NADPH are important biological cofactors for enzymes that are fundamental for various biological processes, such as energy metabolism, mitochondrial function, oxidative stress, immunological functions, and cell death [72]. Antioxidant drugs that could change cellular NAD(P)H concentrations have been effective in diseases such as aging, inflammation, neural degeneration, and cancer [73, 74]. Many tetrazolium compounds can be reduced by NADH and NADPH to produce colored formazin and have been developed commercially for cell vitality assay, as shown in **Table 4** [75].

Triphenyl tetrazolium chloride (TTC) is a lipophilic and light-sensitive compound, and the TTC assay was developed back in 1894 for seed viability tests and is currently a traditional method for brain live-dead neuron evaluation after ischemic stroke [76]. MTT is the most commonly used reagent for cell proliferation or cell toxicity assays, but its formazan is water-insoluble and requires an additional DMSO solubilization step for its quantification at 540 nm [77]. WST-8 is a water-soluble tetrazolium salt, and its brown-colored formazan can be measured directly by UV absorbance at 450 nm. Because of its simple protocol, the WST-8 (CCK-8) assay has become a popular cell vitality method [78–80]. However, WST-8 is not very stable especially under the reduced condition, so EZMTT was developed for drug-induced proliferation assay [81]. EZMTT is another water-soluble tetrazolium salt which is

Tetrazolium	Structural formula	Solubility (tetrazolium/formazin)	Color (tetrazolium/formazin)	Cytotoxicity	Reagent stability
MTT	a	Soluble/insoluble	Yellow/purple	**	**
XTT	e	Soluble/soluble	Yellow/orange	****	****
MTS	i	Soluble/soluble	Yellow/purple	**	****
WST-1	l	Soluble/soluble	Yellow/orange	*	****
CCK-8/WST-8	t	Soluble/soluble	Yellow /orange OD 450 nm	*	***
EZMTT	o	Soluble/soluble	Yellow/orange OD 450 nm	Essentially nontoxic	**

less *** the most*

Table 4.
Comparison of MTT, XTT, MTS, WST-1, CCK-8, and EZMTT.

less toxic than also, EZMTT reagent showed excellent stability and signal of background ratio. Effort has been made to develop the EZMTT to greatly enhance the sensitivity and precision of the drug-induced proliferation assay.

In summary, these cell metabolism-based assays are commonly used as endpoint assays for IC_{50} measurement to rank the potency of a drug. However, the world crisis in drug resistance in infectious disease and cancer called for a deeper look at the drug-induced proliferation assays. Since drug resistance develops owing to a small fraction of the cell population that is resistant to the drug, a sensitive method for detecting partial drug resistance is very important in preventing the occurrence of drug resistance. Another approach is to measure not only the drug potency by IC_{50} values but also the drug efficacy by the drug-induced proliferation rate (DIP) [15]. However, to minimize the experimental error in detecting minor growth, precise DIP rate measurement in HTS mode requires the availability of a continuous assay that can track the cell proliferation from the same samples.

6. Continuous assay

Continuously tracking the cell growth is important for accurate assessment of drug effects and/or growth condition changes. Even though various endpoint assays [75, 82, 83] can be terminated at various time point to obtain the time-dependent cell proliferation curve, the procedures are labor-intensive and have high experimental error. Therefore, a nondestructive continuous assay is highly desirable and critical for the precise evaluation of drug potency and efficacy. Besides the traditional turbidimetric assay by spectrophotometry that has been used for years in microbial assays, four new technologies have been developed that can be used for continuous assays.

6.1 High-content analysis

High-content analysis is a cell imaging and analysis system which includes automatic high-speed microscopic imaging, fully automatic image analysis, and data management. Through snapshot cellular microscopic imaging of a 96-well plate followed by synchronous analysis, the high-content analysis, such as the CloneSelectTM imaging system, accurately measures the cell number without any cell damage. Compared with the MTT method, the experimental deviation of dose pharmacodynamic curve obtained by CloneSelectTM imaging system is smaller and more reproducible. Therefore, high-content analysis has become a reliable choice for big pharmaceutical companies to evaluate drug antiproliferation effects in high-throughput screening, although mostly for attached mammalian cells.

6.2 Electrical impedance technology

Electrical impedance technology-based real-time cellular analysis (RTCA) [84–86] and Epic BenchTop optical biosensors methods [87, 88] have been used in measuring bacterial growth. However, the methods require cells to be cultured on the working electrode or a sensor array of complex structures. The methods [89] cannot detect changes in the cells themselves, so the cell damage caused by drugs and the understanding of the mechanism of drug action are difficult to assess accurately; this inevitably brings detection error and interference. In addition, the requirement of complex laboratory infrastructure further limits their utility.

6.3 Raman spectroscopy analysis

Raman spectroscopy provides a spectroscopic fingerprint of a substance. Based on the difference in monochromatic light with vibrational modes, Raman spectroscopy can be used for qualitative and quantitative measures of the changes in biochemical composition. For example, Raman spectroscopy was used as a noninvasive method to distinguish cells at different stages in the cell cycle [90]; to identify living cells from dead cells [91–95]; to image cellular organelles [96]; to track drug distribution [97] and metabolism [91]; to monitor cell apoptosis [94], death, and cytotoxicity [92, 95]; and to study cell responses to external stimuli [97–101]. However, the analysis of Raman results requires expertise in identifying the spectroscopic fingerprint of a substance.

6.4 EZMTT dye-based cell proliferation analysis

The EZMTT dye [81] was initially designed to overcome the stability issue of WST-8 (CCK-8) reagent which can cause false positives in the presence of antioxidants such as BME (**Figure 5A**) or EGCG. Later, the EZMTT dye was found to be essentially nontoxic and stable in various media [102]. After a single dye addition (**Figure 5B**), the EZMTT method showed linear dose–response to cell numbers and higher signal to background ratio than other relevant methods (**Figure 5C**). The IC_{50} values measured by the EZMTT method are precise and essentially the same as the other methods (**Figure 5D**). Interestingly, when both EZMTT and WST-8 (CCK-8) methods were applied to track the growth of

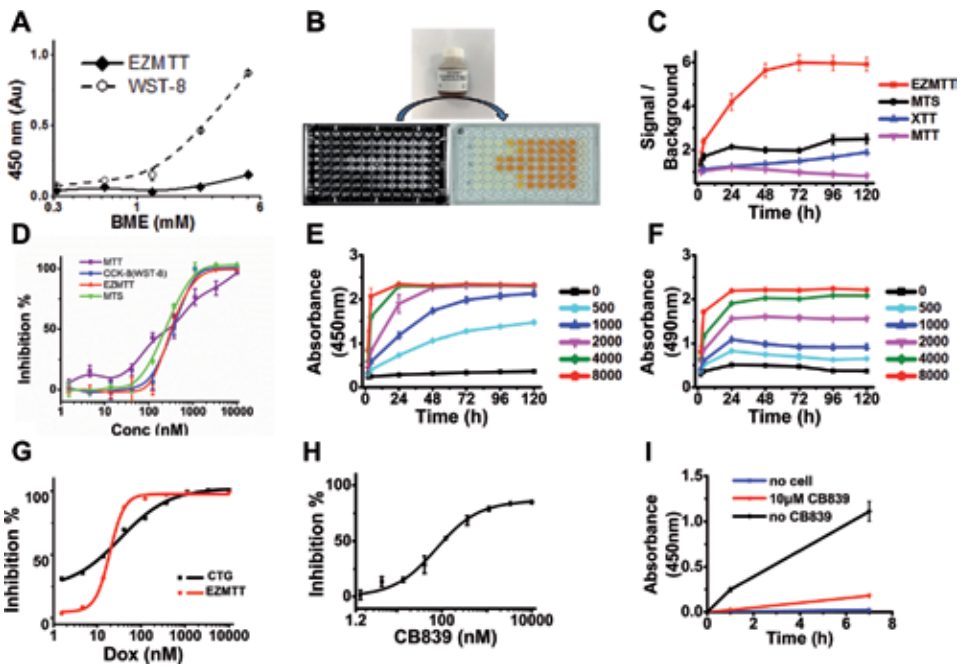

Figure 5.
EZMTT assays: (A) EZMTT dye is stable in the presence of up to 6 mM BME. (B) One step addition of the EZMTT dye allows sensitive measurement of cell growth. (C) Comparison of the signal to background ratio of various tetrazo-based assay (MTS, MTT, CCK-8, EZMTT) in the presence of the same amount of A549 cancer cells. (D) Essentially the same IC_{50} values were obtained from the MTS, MTT, CCK-8, or EZMTT-based assays. (E) Cell growth followed by the EZMTT method. (F) Cell growth followed by the WST-8 method. (G) For a good inhibitor, essentially the same IC_{50} values were obtained from the CTG or EZMTT-based assays. (H) CB839 could only achieve up to 80% inhibtion; (I) the DIP rate of CB839.

A549 lung cancer cells, WST-8 (CCK-8)-treated cells stopped growth in 1 day, whereas the EZMTT-treated cells could grow till saturation (**Figure 5E** and **F**) and allowed easy determination of the cell density and the doubling time in the exponential phase.

In addition, when the EZMTT method was used to track the drug-induced proliferation (DIP) rate changes, the EZMTT method demonstrated high sensitivity and reliability in detecting drug resistance. **Figure 5H** and **I** compares the dose–response curve obtained by the CellTiter-Glo (CTG) method and the EZMTT method. For a sensitive inhibitor, both methods showed essentially the same IC_{50} and % inhibition (**Figure 5H**). When a partial inhibitor is tested, both methods showed essentially the same EC_{50} values, but % inhibition was lower in the EZMTT method, because the partially inhibited cells are still growing (**Figure 5I**). Recently, several KGA allosteric inhibitors were rediscovered as partial inhibitors. For example, CB839 had shown 100% inhibition in CTG assay [103], whereas the EZMTT assay showed approximately 80% inhibition [104], and this is further confirmed by the (DIP) rate measurement. At the steady state, 10 μM CB839 did not completely inhibit cancer cell growth. Interestingly, when 64 nM CB839 and 24 μM ebselen were used in combination, synergistic effects were observed; even though individual compounds only showed partial inhibition, when used in combination, nearly complete inhibition of cancer cell growth was observed [105].

As a new assay format, EZMTT showed powerful applications in cell growth analysis, clinical diagnosis of trivial drug resistance, precise medicine for drug combination, and cost-effective drug discovery by building better correlation between the in vitro cell-based assay and in vivo animal models.

7. Conclusions

Cell proliferation assays are widely used in molecular biology, tumor biology, pharmacology, and pharmacokinetics. It is important in studying not only the basic biological characteristics of cells but also a basic method for analyzing cell states and studying genetic traits. The increase of cell numbers can be simply measured by manual counting of cells under microscopy, using the dye exclusion method (i.e., trypan blue) to count only viable cells. Less fastidious, scalable methods include the use of cytometers; especially the flow cytometry allows combining cell counts ("events") with other specific parameters such as fluorescent probes for membranes, cytoplasm, or nuclei which allows distinguishing dead/viable cells, cell types, cell differentiation, and expression of a biomarker such as Ki67. Beside counting the increasing number of cells, cells can also be assessed based on the metabolic activity, such as the CFDA-SE or calcein-AM method measures not only the membrane functionality (dye retention) but also the functionality of cytoplasmic enzymes (esterases). Also, the MTT-type assays or the resazurin assay (fluorimetric) measures the mitochondrial redox potential. Most of these assays are endpoint assays and may or may not correlate well with the cell proliferation, depending on cell growth conditions, populations of different cells, drug interferences, or toxicity. For precise evaluation of drug-induced proliferation rate changes, a continuous assay is highly desirable. Among various assay formats, the EZMTT dye showed initial promise in precise and sensitive detection of partial inhibition which is the cause of worldwide crisis in drug resistance, and the EZMTT method is expected to provide valuable information for exploring the pathogenesis of the disease, diagnosing the disease, and treating the disease.

Acknowledgements

We appreciate financial support from Natural Science foundation of Zhejiang Province (Grant LY19H300002), Hangzhou Chuying grant award (H1160492), Zhejiang University of Technology (414800129), and Hangzhou qinglan grant award (H1160494).

Conflict of interest

The authors declare no competing interests or other interests that might be perceived to influence the results and/or discussion reported in this paper.

Notes/thanks/other declarations

We thank publishers, Cancer Letter and Experimental Cell Research, for permission of using **Figures 3** and **4**.

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References

[1] Neumann EK, Do TD, Comi TJ, et al. Exploring the fundamental structures of life: non-targeted, chemical analysis of single cells and subcellular structures. Angewandte Chemie-International Edition. 2019;**58**(28):9348-9364

[2] Shestopaloff YK. Why cells grow and divide? General growth mechanism and how it defines cells' growth, reproduction and metabolic properties. Biophysical Reviews and Letters. 2015;**10**(4):48-84

[3] Ren Y, Ji Y, Teng L, et al. Using Raman spectroscopy and chemometrics to identify the growth phase of *Lactobacillus casei* Zhang during batch culture at the single-cell level. Microbial Cell Factories. 2017;**16**(1):233-242

[4] Szymański P, Markowicz M, Mikiciuk-Olasik E. Adaptation of high-throughput screening in drug discovery-toxicological screening tests. International Journal of Molecular Sciences. 2011;**13**(12):427-452

[5] Pariente JL, Kim BS, Atala A. In vitro biocompatibility assessment of naturally derived synthetic biomaterials using normal human urothelial cells. Journal of Biomedical Materials Research. 2015;**55**(1):33-39

[6] Widlak W. Cell division. In: Molecular Biology. Berlin Heidelberg: Springer; 2013

[7] Margolin W. Binary Fission in Bacteria. In: eLS. John Wiley & Sons, Ltd.; 2014

[8] Walker MR, Rapley R. Distribution of nucleic acids within eukaryotic cells. In: Route Maps in Gene Technology. Wiley Blackwell; 2009:30-31

[9] Petronczki M, Siomos MF, Nasmyth K. Un ménage à quatre: The molecular biology of chromosome segregation in meiosis. Cell. 2003;**112**(4):423-440

[10] Humphrey T, Enoch T. Cell-cycle checkpoints. Keeping mitosis in check. Current Biology. 1995;**5**(4):376-379

[11] Dive C, Gregory CD, Phipps DJ, et al. Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. Biochimica et Biophysica Acta. 1992;**1133**(3):275-285

[12] Reed JC. Dysregulation of apoptosis in cancer. The Cancer Journal from Scientific American. 1998;**4**(9):S8

[13] Dienstmann R, Jang IS, Bot B, et al. Database of genomic biomarkers for cancer drugs and clinical targetability in solid tumors. Cancer Discovery. 2015;**5**(2):118-123

[14] Housman G, Byler S, Heerboth S, et al. Drug resistance in cancer: An overview. Cancers. 2014;**6**(3):1769-1792

[15] Harris LA, Frick PL, Garbett SP, et al. An unbiased metric of antiproliferative drug effect in vitro. Nature Methods. 2016;**13**(6):497-500

[16] Kodack DP, Farago AF, Dastur A, et al. Primary patient-derived cancer cells and their potential for personalized cancer patient care. Cell Reports. 2017;**21**(11):3298-3309

[17] Holohan C, Van Schaeybroeck S, Longley DB, et al. Cancer drug resistance: An evolving paradigm. Nature Reviews Cancer. 2013;**13**(10):714-726

[18] Gemryd P, Lundquist PG, Tytor M, et al. Prognostic significance of DNA ploidy in mucoepidermoid carcinoma. European Archives of Oto-Rhino-Laryngology. 1997;**254**(4):180-185

[19] Foss S, Berner A, Jacobsen AB, et al. Clinical significance of DNA ploidy and

S-phase fraction and their relation to p53 protein, c-erbB-2 protein and HCG in operable muscle-invasive bladder cancer. British Journal of Cancer. 1993;**68**(3):572-578

[20] Beccia MR, Biver T, Pardini A, et al. The fluorophore 4′,6-Diamidino-2-phenylindole (DAPI) induces DNA folding in long double-stranded DNA. Chemistry-An Asian Journal. 2012;**7**(8):1803-1810

[21] Poot M, Kavanagh TJ, Kang HC, et al. Flow cytometric analysis of cell cycle-dependent changes in cell thiol level by combining a new laser dye with hoechst 33342. Cytometry. 1991;**12**(2):184-187

[22] Lehtinen J, Nuutila J, Lilius EM. Green fluorescent protein-propidium iodide (GFP-PI) based assay for flow cytometric measurement of bacterial viability. Cytometry. 2004;**60A**(2):165-172

[23] Bellehumeur C, Boyle B, Charette SJ, et al. Propidium monoazide (PMA) and ethidium bromide monoazide(EMA) improve DNA array and high-throughput sequencing of porcine reproductive and respiratory syndrome virus identification. Journal of Virological Methods. 2015;**222**(6):182-191

[24] Wang L, Sun J, Horvat M, et al. Evaluation of MTS, XTT, MTT and 3 HTdR incorporation for assessing hepatocyte density, viability and proliferation. Methods in Cell Science. 1996;**18**(3):249-255

[25] Silva MS, Muñoz PAM, Armelin HA, et al. Differences in the detection of BrdU/EdU incorporation assays alter the calculation for G1, S, and G2 phases of the cell cycle in trypanosomatids. Journal of Eukaryotic Microbiology. 2017;**64**(6):756-770

[26] Bradford JA, Clarke ST. Dual-pulse labeling using 5-ethynyl-2′-deoxyuridine (EdU) and 5-bromo-2′-deoxyuridine (BrdU) in flow cytometry. In: Robinson JP et al., editors. Current Protocols in Cytometry. 2011;7:7-38

[27] Bruno S, Darzynkiewicz Z. Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. Cell Proliferation. 1992;**25**(1):31-40

[28] Darzynkiewicz Z, Zhao H, Zhang S, et al. Initiation and termination of DNA replication during S phase in relation to cyclins D1, E and A, p21WAF1, Cdt1 and the p12 subunit of DNA polymerase δ revealed in individual cells by cytometry. Oncotarget. 2015;**6**(14):11735-11750

[29] Baple EL, Chambers H, Cross HE, et al. Hypomorphic PCNA mutation underlies a human DNA repair disorder. Journal of Clinical Investigation. 2014;**124**(7):3137-3146

[30] Cowell IG, Papageorgiou N, Padget K, et al. Histone deacetylase inhibition redistributes topoisomerase IIb from heterochromatin to euchromatin. Nucleus. 2011;**2**(1):61-71

[31] Hendzel MJ, Wei Y, Mancini MA, et al. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric hetero-chromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma (Berlin). 1997;**106**(6):348-360

[32] Darzynkiewicz Z, Juan G, Li X, et al. Cytometry in cell necrobiology: Analysis of apoptosis and accidental cell death (necrosis). Cytometry. 2015;**27**(1):1-20

[33] Elsässer A, Vogt AM, Nef H, et al. Human hibernating myocardium is jeopardized by apoptotic and autophagic cell death. Journal of the

American College of Cardiology. 2004;**43**(12):2191-2199

[34] Zucker RM, Hunter S, Rogers JM. Confocal laser scanning microscopy of apoptosis in organogenesis-stage mouse embryos. Cytometry. 1998;**33**(3):348-354

[35] Muratori M, Forti G, Baldi E. Comparing flow cytometry and fluorescence microscopy for analyzing human sperm DNA fragmentation by TUNEL labeling. Cytometry. Part A: The Journal of the International Society for Analytical Cytology. 2008;**73A**(9):785-787

[36] Asher E, Payne CM, Bernstein C. Evaluation of cell death in EB V-transformed lymphocytes using agarose gel electrophoresis, light microscopy and electron microscopy: II. Induction of non-classic apoptosis ("para-apoptosis") by tritiated thymidine. Leukemia & Lymphoma. 1995;**19**(1-2):13

[37] Gunawan F, Nishihara J, Liu P, et al. Comparison of platform host cell protein ELISA to process-specific host cell protein ELISA. Biotechnology and Bioengineering. 2018;**115**:382-389

[38] Sureda FX, Escubedo E, Gabriel C, et al. Mitochondrial membrane potential measurement in rat cerebellar neurons by flow cytometry. Cytometry. 1997;**28**(1):74-80

[39] Engeland MV, Nieland LJW, Ramaekers FCS, et al. Annexin V-affinity assay: A review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry. 1998;**31**(1):1-9

[40] Koç E, Çelik-Uzuner S, Uzuner U, et al. The detailed comparison of cell death detected by annexin V-PI counterstain using fluorescence microscope, flow cytometry and automated cell counter in mammalian and microalgae cells. Journal of Fluorescence. 2018;**28**(6):1393-1404

[41] Zhang C, Wu R, Zhu H, et al. Enhanced antitumor activity by the combination of TRAIL/Apo-2L and combretastatin A-4 against human colon cancer cells via induction of apoptosis in vitro and in vivo. Cancer Letters. 2011;**302**(1):11-19

[42] Jiang X, Wang X. Cytochrome C-mediated apoptosis. Annual Review of Biochemistry. 2004;**73**(73):87-106

[43] Lin L, Deng W, Tian Y, et al. Lasiodin inhibits proliferation of human nasopharyngeal carcinoma cells by simultaneous modulation of the Apaf-1/caspase, AKT/MAPK and COX-2/NF-κB signaling pathways. PLoS One. 2014;**9**(5):e97799

[44] Bucevičius J, Lukinavičius G, Gerasimaitė R. The use of hoechst dyes for DNA staining and beyond. Chem. 2018;**6**(2):18-29

[45] Gibson B, Wilson DJ, Feil E, et al. The distribution of bacterial doubling times in the wild. Proceedings of The Royal Society B-Biological Sciences. 2018;**285**(1880):9

[46] Menetrez MY. An overview of algae biofuel production and potential environmental impact. Environmental Science & Technology. 2012;**46**(13):7073-7085

[47] Väyrynen JP, Vornanen JO, Sajanti S, et al. An improved image analysis method for cell counting lends credibility to the prognostic significance of T cells in colorectal cancer. Virchows Archiv. 2012;**460**:455-465

[48] Kaur C, Pal I, Saini S, et al. Comparison of unbiased stereological estimation of total number of cresyl violet stained neurons and parvalbumin positive neurons in the adult human

spiral ganglion. Journal of Chemical Neuroanatomy. 2018;**93**:30-37

[49] Lodeiro P, Achterberg EP, El-Shahawi MS. Detection of silver nanoparticles in seawater at ppb levels using UV–visible spectrophotometry with long path cells. Talanta. 2017;**164**:257-260

[50] Eltringham I, Pickering J, Gough H, et al. Comparison of mycobacterial growth indicator tube with culture on RGM selective agar for detection of mycobacteria in sputum samples from patients with cystic fibrosis. Journal of Clinical Microbiology. 2016;**54**(8):2047-2050

[51] Wilson ML, Weinstein MP, Reimer LG, et al. Controlled comparison of the BacT/alert and BACTEC 660/730 nanradiometric blood culture systems. Journal of Clinical Microbiology. 1992;**30**(2):323-329

[52] Pina-Vaz C, Costa-de-Oliveira S, Rodrigues AG. Safe susceptibility testing of mycobacterium tuberculosis by flow cytometry with the fluorescent nucleic acid stain SYTO 16. Journal of Medical Microbiology. 2005;**54**(1):77-81

[53] Rychtecky P, Znachor P, Nedoma J. Spatio-temporal study of phytoplankton cell viability in a eutrophic reservoir using SYTOX green nucleic acid stain. Hydrobiologia. 2014;**740**(1):177-189

[54] Andras SC, Hartman TPV, Alexander J, et al. Combined PI–DAPI staining (CPD) reveals NOR asymmetry and facilitates karyotyping of plant chromosomes. Chromosome Research. 2000;**8**(5):387-391

[55] Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nature Protocols. 2006;**1**(3):1112-1116

[56] Askenasy N, Farkas DL. Optical imaging of PKH-labeled hematopoietic cells in recipient bone marrow in vivo. Stem Cells. 2002;**20**(6):501-513

[57] Bhowmik BB, Basu S, Ray D. Photophysical studies of 3,3′ dioctadecyloxacarbocyanine dye in model biological membranes and different solvents. Chemistry & Physics of Lipids. 2001;**109**(2):175-183

[58] Yumoto K, Berry JE, Taichman RS, et al. A novel method for monitoring tumor proliferation in vivo using fluorescent dye DiD. Cytometry Part A. 2014;**85**(6):548-555

[59] Edgardo J, Liaudat JP, Medeot D, et al. Monitoring succinoglycan production in single Sinorhizobium meliloti, cells by Calcofluor white M2R staining and time-lapse microscopy. Carbohydrate Polymers. 2017;**181**:918-922

[60] Scornik FS, Bucciero RS, Wu Y, et al. DiBAC4(3) hits a "sweet spot" for the activation of arterial large-conductance Ca2+–activated potassium channels independently of the β1-subunit. AJP: Heart and Circulatory Physiology. 2013;**304**(11):H1471-H1482

[61] Voytik-Harbin SL, Brightman AO, Waisner B, et al. Application and evaluation of the alamarblue assay for cell growth and survival of fibroblasts. In Vitro Cellular & Developmental Biology Animal. 1998;**34**(3):239-246

[62] O'brien J, Orton T, Wilson I, et al. Investigation of the Alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. European Journal of Biochemistry. 2010;**267**(17):5421-5426

[63] Mueller D, Tascher G, Damm G, et al. Real-time in situ viability assessment in a 3D bioreactor with liver;cells using resazurin assay. Cytotechnology. 2013;**65**(2):297-305

[64] Crouch SPM, Kozlowski R, Slater KJ, et al. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. Journal of Immunological Methods. 1993;**160**(1):81

[65] Nilsson LE, Molin O, Anséhn S. Bioluminescent assay of bacterial ATP for rapid detection of bacterial growth in clinical blood cultures. Journal of Bioluminescence and Chemiluminescence. 1989;**3**(3):101-104

[66] Angulo R, Fulcher DA. Measurement of Candida-specific blastogenesis: Comparison of carboxyfluorescein succinimidyl ester labelling of T cells, thymidine incorporation, and CD69 expression. Cytometry Part A. 2015;**34**(3):143-151

[67] Parish CR. Fluorescent dyes for lymphocyte migration and proliferation studies. Immunology and Cell Biology. 1999;**77**(6):499-508

[68] Nayla J, Claus RA, Katja D, et al. Comparative suitability of CFDA-SE and rhodamine 6G for in vivo assessment of leukocyte-endothelium interactions. Journal of Biophotonics. 2014;**7**(6):369-375

[69] Kai Z, Kunpeng P, Xinyi W. Isolation and transplantation of corneal endothelial cell-like cells derived from in-vitro-differentiated human embryonic stem cells. Stem Cells & Development. 2014;**23**(12):1340-1354

[70] Braut-Boucher F, Pichon J, Rat P, et al. A non-isotopic, highly sensitive, fluorimetric, cell-cell adhesion microplate assay using calcein AM-labeled lymphocytes. Journal of Immunological Methods. 1995;**178**(1):41-51

[71] Legrand C, Bour JM, Jacob C, et al. Lactate dehydrogenase (LDH) activity of the number of dead cells in the medium of cultured eukaryotic cells as marker. Journal of Biotechnology. 1992;**25**(3):231-243

[72] Ying W. NAD+/NADH and NADP+/NADPH in cellular functions and cell death: Regulation and biological consequences. Antioxidants & Redox Signaling. 2008;**10**(2):179-206

[73] Tsang YH, Dogruluk T, Tedeschi PM, et al. Functional annotation of rare gene aberration drivers of pancreatic cancer. Nature Communications. 2016;**7**:10500-10510

[74] Halliwell B. Drug antioxidant effects. A basis for drug selection? Drugs. 1991;**42**(4):569-605

[75] Ramadhin C, Pillay B, Olaniran AO. Cell-based assays for IGF-I bioactivity measurement: Overview, limitations and current trends. Growth Factors. 2014;**32**(3-4):130

[76] Otero AJ, Rodríguez I, Falero G. 2,3,5-triphenyl tetrazolium chloride (TTC) reduction as exponential growth phase marker for mammalian cells in culture and for myeloma hybridization experiments. Cytotechnology. 1991;**6**(2):137-142

[77] Vistica DT, Skehan P, Scudiero D, et al. Tetrazolium-based assays for cellular viability: A critical examination of selected parameters affecting formazan production. Cancer Research. 1991;**51**(10):2515-2520

[78] Mariana DNH, Eziefula AC, Othieno L, et al. Tools for mass screening of G6PD deficiency: Validation of the WST8/1-methoxy-PMS enzymatic assay in Uganda. Malaria Journal. 2013;**12**(1):210-210

[79] Naoi T, Shibuya N, Inoue H, et al. The effect of tert-butylhydroquinone-induced oxidative stress in MDBK cells using XTT assay: Implication of tert-butylhydroquinone-induced NADPH generating enzymes. Journal

of Veterinary Medical Science. 2010;**72**(3):321-326

[80] Tiwari K, Wavdhane M, Haque S, et al. A sensitive WST-8-based bioassay for PEGylated granulocyte colony stimulating factor using the NFS-60 cell line. Pharmaceutical Biology. 2015;**53**(6):849-854

[81] Zhang W, Zhu M, Wang F, et al. Mono-sulfonated tetrazolium salt based NAD(P)H detection reagents suitable for dehydrogenase and real-time cell viability assays. Analytical Biochemistry. 2016;**509**:33-40

[82] Adan A, Kiraz Y, Baran Y. Cell proliferation and cytotoxicity assays. Current Pharmaceutical Biotechnology. 2016;**17**(14):1213-1221

[83] Riss TL, Moravec RA. Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. Assay and Drug Development Technologies. 2004;**2**(1):51-62

[84] Vega-Avila E, Pugsley MK. An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells. Proceedings of the Western Pharmacology Society. 2011;**54**:10-14

[85] Atienza JM, Zhu J, Wang X, et al. Dynamic monitoring of cell adhesion and spreading on microelectronic sensor arrays. Journal of Biomolecular Screening. 2005;**10**(8):795-805

[86] Giaever I, Keese CR. Monitoring fibroblast behavior in tissue culture with an applied electric field. Proceedings of the National Academy of Sciences of the United States of America. 1984;**81**(12):3761-3764

[87] Keese CR, Bhawe KJ, Giaever I. Real-time impedance assay to follow the invasive activities of metastatic cells in culture. BioTechniques. 2002;**33**(4):842-844

[88] Caide X, Bernard L, Geoffrey S, et al. Assessment of cytotoxicity using electric cell-substrate impedance sensing: Concentration and time response function approach. Analytical Chemistry. 2002;**74**(22):5748-5753

[89] Xiao C, Luong JH. On-line monitoring of cell growth and cytotoxicity using electric cell-substrate impedance sensing (ECIS). Biotechnology Progress. 2010;**19**(3):1000-1005

[90] Wang L, Wang L, Yin H, et al. Real-time, label-free monitoring of the cell cycle with a cellular impedance sensing chip. Biosensors & Bioelectronics. 2010;**25**(5):990-995

[91] Keating ME, Byrne HJ. Raman spectroscopy in nanomedicine: Current status and future perspective. Nanomedicine. 2013;**8**(8):1335-1351

[92] Chernenko T, Sawant RR, Miljkovic M, et al. Raman microscopy for non-invasive imaging of pharmaceutical nanocarriers: Intracellular distribution of cationic liposomes of different composition. Molecular Pharmaceutics. 2012;**9**(4):930-936

[93] Dorney J, Bonnier F, Garcia A, et al. Identifying and localizing intracellular nanoparticles using Raman spectroscopy. The Analyst. 2012;**137**(5):1111

[94] El-Mashtoly SF, Petersen D, Yosef HK, et al. Label-free imaging of drug distribution and metabolism in colon cancer cells by Raman microscopy. Analyst. 2014;**139**(5):1155-1161

[95] Hartmann K, Becker-Putsche M, Bocklitz T, et al. A study of docetaxel-induced effects in MCF-7 cells by means of Raman microspectroscopy.

Analytical & Bioanalytical Chemistry. 2012;**403**(3):745-753

[96] Nawaz H, Garcia A, Meade AD, et al. Raman micro spectroscopy study of the interaction of vincristine with A549 cells supported by expression analysis of bcl-2 protein. The Analyst. 2013;**138**(20):6177

[97] Heath JR, Ribas A, Mischel PS. Single-cell analysis tools for drug discovery and development. Nature Reviews. Drug Discovery. 2016;**15**(3):204-216

[98] Deng R, Qu H, Liang L, et al. Tracing the therapeutic process of targeted aptamer/drug conjugate on cancer cells by surface-enhanced Raman scattering spectroscopy. Analytical Chemistry. 2017;**89**(5):2844-2851

[99] Oh BR, Chen P, Nidetz R, et al. Multiplexed nanoplasmonic temporal profiling of T-cell response under immunomodulatory agent exposure. Acs Sensors. 2016;**1**(7):941-948

[100] El-Mashtoly SF, Yosef HK, Dennis P, et al. Label-free Raman spectroscopic imaging monitors the integral physiologically relevant drug responses in cancer cells. Analytical Chemistry. 2015;**87**(14):7297-7304

[101] Farhane Z, Bonnier F, Howe O, et al. Doxorubicin kinetics and effects on lung cancer cell lines using in vitro Raman micro-spectroscopy: Binding signatures, drug resistance and DNA repair. Journal of Biophotonics. 2018;**11**(1):14

[102] Zhu M, Fang J, Zhang J, et al. Biomolecular interaction assays identified dual inhibitors of glutaminase and glutamate dehydrogenase that disrupt mitochondrial function and prevent growth of cancer cells. Analytical Chemistry. 2017;**89**:1689-1696. DOI: 10.1021/acs.analchem.6b03849

[103] Gross MI, Demo SD, Dennison JB, et al. Antitumor activity of the glutaminase inhibitor CB-839 in triple-negative breast cancer. Molecular Cancer Therapeutics. 2014;**13**:890-901

[104] Chen Z, Li D, Xu N, et al. Novel 1,3,4-selenadiazole-containing kidney-type glutaminase inhibitors showed improved cellular uptake and antitumor activity. Journal of Medicinal Chemistry. 2019;**62**(2):589-603. DOI: 10.1021/acs.jmedchem.8b01198

[105] Ruan JJ, Yu Y, Hou W, et al. Kidney type glutaminase inhibitor hexylselen selectively kills cancer cells via a three-pronged mechanisms. ACS Pharmacology & Translational Science. 2019;**2**(1):18-30

Chapter 5

Ion Homeostasis Response to Nutrient-Deficiency Stress in Plants

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Abstract

A crucial feature of plant performance is its strong dependence on the availability of essential mineral nutrients, affecting multiple vital functions. Indeed, mineral-nutrient deficiency is one of the major stress factors affecting plant growth and development. Thereby, nitrogen and potassium represent the most abundant mineral contributors, critical for plant survival. While studying plant responses to nutrient deficiency, one should keep in mind that mineral nutrients, along with their specific metabolic roles, are directly involved in maintaining cell ion homeostasis, which relies on a finely tuned equilibrium between cytosolic and vacuolar ion pools. Therefore, in this chapter we briefly summarize the role of the ion homeostasis system in cell responses to environmental deficiency of nitrate and potassium ions. Special attention is paid to the implementation of plant responses via NO_3^- and K^+ root transport and regulation of ion distribution in cell compartments. These responses are strongly dependent on plant species, as well as severity and duration of nutrient deficiency.

Keywords: nutrient deficiency, ion homeostasis, nitrate, potassium, ion transport mechanisms, vacuolar and cytosolic ion pools

1. Introduction

Plant growth and development often depend on various biotic and abiotic stressors. In particular, alterations in environmental conditions have a direct impact on the nutrient uptake and assimilation in plants [1]. In this context, a surplus and even more so a deficiency of essential macronutrients in soils represents one of the most common stress types, with nitrogen (N), potassium (K) and phosphorous (P) being the most relevant ones. These macronutrients are directly involved in multiple metabolic pathways and physiological responses, acting as structural constituents of vital metabolites, playing a key role in osmotic regulation and cellular permeability, being critical for proper growth and development [2]. Currently, it is often assumed that due to the existence of fast recycling mechanisms, macronutrient availability does not limit plant growth in natural, uncultivated systems. However, due to a widely spread overexploitation

of soils, it might be the case in the modern agricultural practice [3]. In particular, deficiency of nitrogen (N) and potassium (K) is quite common in developing and least developed countries, especially in rice and wheat production in Asia, Africa and Central and South America [4]. Such nutrient deficiency eventually leads to decrease of plant productivity and losses of crop yields. Visual manifestations of stress caused by a deficiency of individual macro- and microelements are well documented [5]. The underlying key physiological processes, affected by mineral deficiencies, are well characterized and include photosynthesis, protein synthesis, primary and secondary metabolism and carbohydrate distribution between source and sink tissues [6–8].

The methods of biochemistry and molecular biology proved to be efficient in disclosing the fine regulatory mechanisms behind ion homeostasis in plants [8]. Thus, the emergence of RNA microarray technology tremendously contributed to the investigation of rapid transcriptional changes associated with mineral imbalance [9–13]. Most of the studies addressing plant responses of ion-transporting systems to deficiencies of K^+ and NO_3^- rely on *Arabidopsis thaliana* [9, 11–13]. The same is true for phosphorus [14, 15], not further detailed here. However, experiments with such crop plants as wheat [16], tomato [17], rice [18], barley, pepper as well as *Mesembryanthemum crystallinum* [19] revealed a pronounced increase in abundance of K^+ transporter transcripts in response to potassium starvation. Similarly, expression of nitrate transporters in wheat roots and leaves [20, 21], sorghum [22] and rice [23, 24] seedlings was enhanced in response to NO_3^- starvation.

The fact, that all mineral nutrients enter the plant in ionic form and, along with involvement in metabolic processes, are crucial for maintaining the cell ion homeostasis seems to be underestimated. Thereby, the existence of cytosolic and vacuolar ion pools needs to be taken into account. These pools are maintained by numerous membrane ion transporters, representing an integrated part of a complex cellular regulatory network [25]. Hence, the specificity of plant responses to nutritional stress may imply a relevant adjustment of systems involved in absorption, transport, distribution, accumulation and remobilization of mineral ions.

Nitrogen and potassium are the most abundant mineral elements in plant nutrition. The principal features of their reception and distribution in plant organs and cells are well studied to date [2]. On the other hand, a lack of these nutrients in soils (especially in the form of NO_3^- and K^+) is quite a common phenomenon [8] that appears to be an appropriate argument for a more thorough analysis of plant responses to NO_3^- and K^+ deficiency given the role of ion homeostasis system in their development.

2. The role of ion homeostasis in the mechanisms of plant responses to nitrate deficiency

2.1 Nitrate deficiency and its effects on plants

Nitrogen is not only the most abundant mineral element in plants [2], but it also contributes signaling [26] and often plays the role of the limiting nutrient for plant growth [27]. However, in aerobic soils, nitrate (NO_3^-) often represents the predominant source of inorganic nitrogen [28, 29]. However, due to its high solubility in soil water, NO_3^- is a highly mobile ion [30, 31]. Moreover, its distribution in soils is heterogeneous; thereby in temperate regions the concentrations of nitrate in soil solution can vary from a few micromoles in nonagricultural soils to several millimoles after fertilization [32, 33].

Nitrogen is known to be one of the major elements contributing to the structure of organic molecules, including proteins, nucleic acids, cofactors and metabolites [2, 13]. Therefore, a sustainable supply of plants with NO_3^- is critically important for plant survival. Indeed, any limitation of nitrate availability affects plant function at different levels—from cellular metabolism to resource allocation, growth and development [2, 13, 34, 35]. The most pronounced visual sign of nitrogen deficiency is chlorosis of older leaves. Since more than half of the total leaf nitrogen is allocated to the photosynthetic apparatus, nitrate shortage results in a marked decrease in the plant photosynthesis [36]. Due to the activity of nitrogen-fixing and nitrifying bacteria, the complete absence of nitrate in soil is rather unlikely. However, this scenario can be simulated in aqueous culture experiments by exclusion of nitrate or other nitrogen sources transformable to nitrate (e.g. urea and ammonium) from the nutrient solution for several days with subsequent re-supplementation [33, 34]. Such experiments proved to be useful to reveal a wide range of responses and multiple mechanisms of plant adaptation to nitrate stress.

Adaptation of plants to the prevailing soil nitrate conditions rely on systemic response mechanisms, finely tuned during millions years of terrestrial plant evolution [37]. According to Miller and Cramer [38], plants respond to nitrate stimuli by adjusting expression of the genes, involved in nitrate transport and assimilation—that is, nitrate transporters and assimilatory enzymes, in parallel with post-translational modifications of these proteins. The simplest model, explaining nitrate sensing in plant root cells, assumes alteration of cytosolic nitrate concentrations in agreement with its external supply [33]. The authors propose that the cytosol represents a finely regulated ionic environment with precise nitrate homeostasis, that is, even minor changes in cytosolic nitrate levels in response to external alterations in nutrient supply might essentially impact on cell signaling [33].

It is well known that nitrate deficiency is closely related to the plant primary metabolism. For example, in *Arabidopsis* leaves, the plant response to nitrate starvation is manifested with the accumulation of sugars and allocation of carbon to roots. This, in turn, results in enhancement of root growth and triggering branching of the lateral roots [8, 13, 39]. This response is important for establishment of root morphology and is accompanied with an increase of root to shoot biomass ratio and in root absorption capacity [40, 41]. As was shown by the RNA microarray techniques, the observed metabolic shifts were underlied by clearly detectable transcriptional changes, which could be mapped to pathways resulting in accumulation of mono- and oligosaccharides, and starch in shoots [8]. For example, enhanced translocation of sucrose from shoots to the roots might indicate an increase in plant root to shoot biomass ratio [8]. Along with the above mentioned fast transcriptional changes, associated with mineral imbalance, the relationship between the cell genome, metabolome and ionome has also become a subject of a special interest [13, 42]. The ionome is usually defined as the mineral nutrient and trace element composition of an organism and represents the inorganic component of cells and organisms [42]. Significant progress has been made in the field of ionomics in the last decade, in which high-throughput elemental profiling is combined with genetics to identify the genes that control the ionome [43]. Since sugars exert metabolite feedback regulation and affect multiple genes involved in photosynthesis, it can be assumed that photosynthesis suppression in nitrogen-deficient plants represents a direct consequence of sugar accumulation in leaves [8]. On the other hand, it needs to be taken into account that primary metabolites play a crucial role in expression regulation of several genes involved in nitrogen consumption and metabolism [44]. Not less important, nitrate itself is involved in regulation of multiple genes assigned to sugar metabolism [9].

2.2 Regulation of NO_3^- transport and mobilization of NO_3^- storage pools in plant responses to nitrate deficiency

Importantly, root growth and branching in response to nitrate deficiency are accompanied with an increase in the number of nitrate transporters in plant roots [31]. It results in enhancement of nitrate influx due to up-regulation of nitrate transporters in the membranes of root cells. Not less important might be improvement of their biochemical properties and increase of nitrogen utilization efficiency within the plant [31, 45].

During the last two decades, multiple nitrate transporters/channels were identified and comprehensively characterized [31, 41, 46]. These proteins belong to at least four different families: nitrate transporter 1/peptide transporter family (NRT1/NPF), nitrate transporter 2 (NRT2) family, chloride channel (CLC) family and slow anion channel-associated homologs (SLAC/SLAH) [31, 46]. Thereby, the complex processes, underlying NO_3^- uptake, translocation and storage in plants, are controlled by fine regulation and crosstalk between these four main types of transporters [47]. This crosstalk gives plants access to sufficient amounts of bioavailable nitrate under changing levels of environmental nitrogen. Among the listed NO_3^- transporters, NRT1.1 (NPF6.3) is one of the best characterized [47]. It was shown to be a dual-affinity transporter [48] with sensing functions [41, 49]. The activity of this protein is regulated via phosphorylation at the threonine T101 by calcineurin B-like (CBL)-interacting protein kinase (CIPK23) [48]. This phosphorylation results in a shift from a low affinity to a high-affinity NO_3^- uptake kinetics [50] with a K_m of approximately 4 mmol/L for the low-affinity phase of uptake and 50 µmol/L for the high-affinity one.

As far as NRT2 is concerned, it functions as a high-affinity nitrate transporter in association with a smaller protein NAR 2 (NRT3), which facilitates targeting of NRT2 to the plasma membrane [31, 32]. Under a limited access to exogenic nitrate, altogether four NRT2 transporters (NRT2.1, NRT2.2, NRT2.4 and NRT2.5) take up approximately 95% of the total amount of this anion, with NRT2.1 and NRT2.2 being the main contributors [51]. In durum wheat plants, NRT2.5 was shown to be mostly up-regulated under the conditions of $NO3^-$ starvation [20]. In sorghum, N stress caused higher abundance of NRT2.2, NRT2.3, NRT2.5 and NRT2.6 transcripts [22].

It is well known that nitrate, as the principal nitrogen source, can be accumulated in leaves and stems of some crop plants, specifically in the plants belonging to the families Chenopodiaceae, Brassicaceae and Asteraceae where nitrate contents can exceed 300 mmol/kg fresh weight or 17–24% of the plant dry weight [52]. Such a strong accumulation of nitrate can be explained by the existence of a small "metabolic" pool of nitrate accessible to nitrate reductase (NR) in cytosol and a large "storage" pool, compartmentally separated from the sites of its metabolism [53]. Later, the predominant identity of this storage compartment was confirmed as the central vacuole of the leaf cell [54]. Further evaluation of cytosol nitrate concentrations using compartmental analysis and cell fractionation techniques provided a solid evidence for the existence of nitrate homeostasis [55] and supported existence of a variable nitrate pool [56]. It was shown that a nitrate/proton ($2NO_3^-/1H^+$) antiporter AtCLCa, localized in the tonoplast, is expressed in both shoots and roots and mediates nitrate accumulation in the vacuole [57] as the most important requirement for maintaining nitrate homeostasis in cells. The major mechanisms prospectively involved in plant responses to nitrate deficiency are summarized in **Figure 1**.

Due to a pronounced success in isolation of pure fractions of barley leaf vacuole vesicles, the storage nitrate pools were found to range from 58% [58] to 99% of the total cellular nitrate content [54]. Indeed, over 90% volume of a mature plant cell is occupied by the vacuole, while the nitrate concentrations in cytoplasm and vacuole

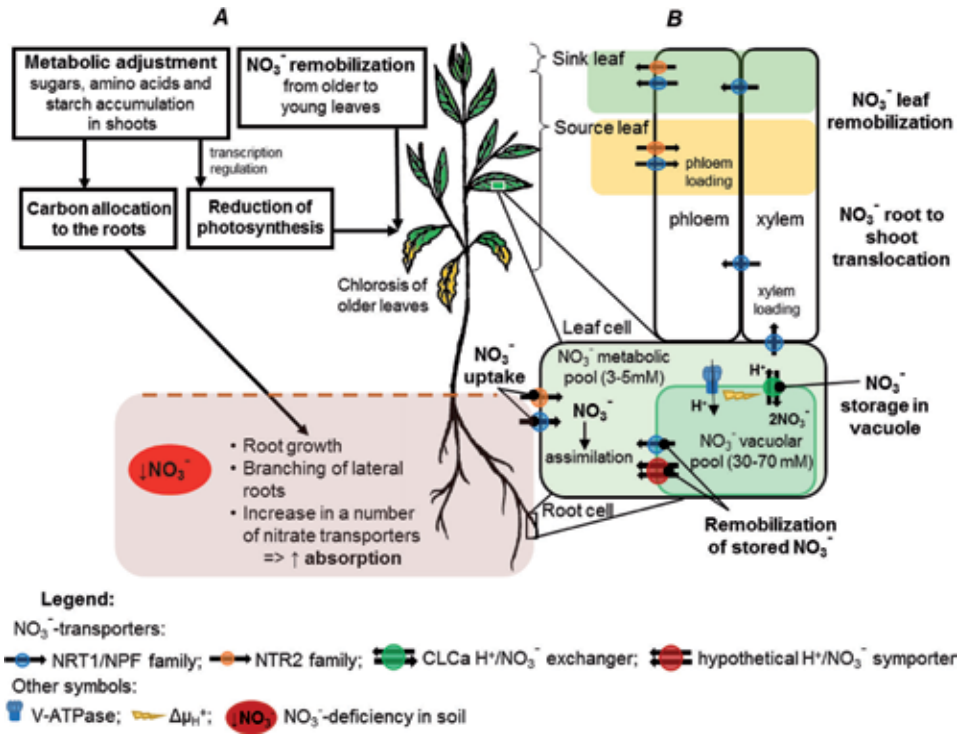

Figure 1.
Schematic view of plant responses to nitrate deficiency. (A) The effect of NO_3^- deficiency on carbon metabolism in plants. The carbon flow from shoots to roots is triggered in response to NO_3^- deficiency in soil, and is underlied by the mechanisms of metabolic adjustment and NO_3^- remobilization. This carbon flow supports root growth and branching, sustaining their adsorption capacity and provides young (sink) leaves with NO_3^-, whereas older (source) leaves undergo chlorophyll degradation. Chlorosis of leaves serves as a key indicator of NO_3^- deficiency. (B) The major transport systems, involved in plant response to NO_3^- deficiency. NO_3^- deficiency results in activation of the plant nitrate transport systems, involved in NO_3^- uptake, accumulation, remobilization in root cell, root cell-to-cell distribution, root to shoot and source to sink leaves translocations.

are generally in the range of 3–5 and 30–70 mmol/L, respectively [52, 59, 60]. For example, when Arabidopsis plants were grown in modified Hoagland solution, containing 4.25 mmol/L NO_3^-, nitrate concentrations in the cytosol of leaf mesophyll cells were 2.8 mmol/L, whereas in vacuoles of the same cells, this value reached 31.8 mmol/L [61]. It is important to notice that nitrate, stored in the vacuole, may be available for assimilation, serving thereby as a reservoir supporting growth for longer periods when external nitrogen supply is limited to only several days [62].

Van der Leij et al. [63] were the first to study the remobilization of vacuolar nitrate in the root cells of barley seedlings during 24 h of nitrate deprivation using double-barrelled nitrate-selective microelectrodes. Their measurements showed much slower remobilization from the vacuoles of cortical cells than those in epidermal cells. The cytosolic nitrate concentrations (activities) in both roots and shoots slowly decreased from 3.7 to 2.9 mmol/L during the first 24 h of the nitrate deprivation, whereas the vacuolar and xylem nitrate activities gradually decreased from 69.6 to 40.2 mmol/L in the same period. In this regard, the authors proposed that the remobilization of vacuolar nitrate can be treated as short term, that is, lasting not more than a few days [63]. It was shown in later studies that nitrate, accumulating in the vacuole, cannot be rapidly transported into the cytoplasm and the transport rates depend on genotypes and differ between individual cultivars [23, 29]. The reuse of NO_3^- in plant tissues to a great extent depends on its transport from the vacuole to the cytoplasm and vice versa [64, 65]. According to Glass et al.

[66], low concentrations of NO_3^- in the cytosol are sufficient to ensure NR activity, whereas relatively high amounts of nitrate in the vacuole only minimally influence the NR activity [66].

Based on the ability to sustain nitrate deficiency, tolerant and sensitive genotypes can be distinguished [51]. At least to some extent, this difference might depend on the rates of nitrate transport across the tonoplast from vacuole into cytoplasm during nitrate starvation and on the ability to restore the vacuolar pool under normal nitrate supply afterwards [52]. Thereby, the tonoplast proton pumps (V-ATPase and V-PPase) play a key role in the distribution of NO_3^- between vacuole and cytoplasm [59] by building an electrochemical transmembrane proton gradient [52, 67]. It was proposed that the NO_3^- transport from the cytoplasm to the vacuole is mainly mediated by the vacuole H^+/ NO_3^- antiport system [68]. Later, it was demonstrated that AtCLCa operates as a NO_3^-/H^+ exchanger with a 2:1 nitrate-proton stoichiometry, building an up to 1:50 nitrate gradient between the two compartments [57]. In this context, the nitrate/proton symport system might be responsible for the remobilization of the vacuolar nitrate [69]. However, the mechanisms behind nitrate efflux from vacuoles to cytoplasm are mostly unknown so far. The tonoplast efflux NO_3^- transporters were not identified as well [31]. However, three members of the NRT1/NPF family, localized in the tonoplast—NPF5.11, NPF5.12 and NPF5.16—have been proposed to be the vacuolar nitrate efflux transporters in Arabidopsis [67]. They are predominantly expressed in the cells of root pericycle and xylem parenchyma, thus acting as important players in modulation of nitrate allocation between roots and shoots and being crucial for plant adaptation to the changing environment [67]. Generally, the vacuole is not only a storage location for nitrate but also impacts on turgor maintenance and osmotic balance [33, 50, 70].

Usually, the highest concentrations of nitrate are found in stems and petioles, whereas the contents of nitrate are lower in leaf blades [50]. According to our own observations, in lettuce and Chinese cabbage, the corresponding differences in nitrate concentrations can reach two- to eightfold under high nitrate supply [71]. In response to a 7-day long nitrate exclusion from the nutrient solution, the nitrate contents decreased primarily in vascular tissues. Essentially this reduction in nitrate contents was accompanied with a pronounced increase in the levels of chloride and anions of organic acids, obviously acting to compensate for nitrate to maintain the required turgor in the vacuoles.

It is logical to assume that the ability of different plants to generate, maintain and mobilize the reserve nitrate pools in vascular tissues is one of the strategies for their survival under adverse conditions of nitrogen deficiency. In particular, nitrate storage pools enable survival of winter cultivars during the periods of nitrate depletion in soils due to intensive rains [32]. This strategy is in agreement with the plant inherited nitrate mobilization and transport from older (source) to younger (sink) leaves during plant ontogenesis or when the external nitrate availability is decreasing [50, 72]. This was confirmed by the results of gene expression analysis. Thus, it was shown that the expression of the AtNRT1.7 gene is induced by nitrogen starvation in the sieve elements/companion cells of the leaf minor veins and facilitates phloem loading in older leaves [73]. Analogously, AtNRT2.4 and AtNRT2.5 are induced by nitrogen starvation and repressed by nitrogen resupply [74]. NRT2.4 is expressed in the phloem of leaf major veins and might retrieve nitrate under nitrogen starvation towards minor veins [75]. In turn, NRT2.5 is expressed in minor veins and, together with NRT2.4, may affect leaf remobilization and phloem transport of nitrate [51]. Moreover, NRT1.11 and NRT1.12, localized in the companion cells of the leaf major veins, were shown to impact on phloem loading, besides playing a role in xylem-to-phloem transfer [76].

Thus, it can be concluded that activation and induction of nitrate transport and mobilization of nitrate from its reserve pools likely are an important component of the cell response to nitrate deficiency.

3. The role of ion homeostasis in the mechanisms of plant responses to potassium deficiency

3.1 Potassium deficiency and its effects on plants

Potassium is the second (after nitrogen) most abundant mineral element in plants [2]. It is the main cation (K^+) in plant cells [77] and is essential for plant growth and adaptation to the environment [78]. In contrast to nitrogen, potassium is not involved in metabolism and remains in ionic form to execute its specific functions in plant cells [79]. Potassium is associated with or directly involved in several physiological processes supporting plant growth and development—photosynthesis, protein and starch biosynthesis; transport of sugars and nutrients; and stomatal closure [77, 80]. Moreover, K^+ was shown to be essential in the activation of 50–60 key enzymes, involved in critical metabolic processes [81, 82], including photosynthesis, oxidative metabolism and protein synthesis [2]. Thus, this cation might be involved in the regulation of metabolite patterns and their relative abundances in higher plants [83].

As mentioned above, the lack of potassium might suppress various enzymatic activities [81, 82], but this can occur only when potassium cytosolic contents decrease due to prolonged K deficiency [81]. Hence, cytosolic K^+ homeostasis is crucial for the central cell metabolism [84], plant growth and adaptation to the environment and must, therefore, be finely controlled [78]. In addition, K^+ plays a crucial role in the establishment of cell turgor and osmoregulation, neutralization/scavenging of anions (e.g. those of organic and inorganic acids), control of cytosolic pH, ion homeostasis and electrical membrane potential [77, 78, 83, 85].

Potassium availability in soil is the main factor, affecting supply of terrestrial plants with this element. Indeed, although potassium is the seventh most abundant element in the Earth crust [86], only a small part of the whole soil potassium pool is present in a form readily available for plants, whereas most of the soil potassium constitutes hardly soluble minerals [87]. Thus, due to the low rates of their solubilization, the concentration of biologically available K^+ in soil solution is rather low and varies between 0.1 and 1.0 mmol/L [82, 88]. Moreover, due to a rapid local depletion at the root surface, in reality these values can be even lower, and supply of the plant with potassium is highly dependent on the rates of its liberation from minerals and transport in soil solution [89]. Both rates are typically relatively slow: growing plants can deplete soil solution to yield potassium concentrations between 1 and 2 μmol/L. On the other hand, this might result in enhancement of potassium release and mobilization in soil [89]. Obviously, the amounts of soil water (i.e. soil water contents) essentially affect the gradients of potassium concentrations on the root surface. Thus, diffusion of K^+ is essentially restricted in dry soil but is significantly increased upon re-supplementation of soil with water [89].

According to the available literature data, under sufficient nutritional supply, the average potassium contents in the most of the plant species vary between 4 and 8% (w/w) in dry matter [84]. However, for multiple other species, the optimal potassium contents are essentially lower and lie in the range of 0.5–2.0% (w/w) [80]. Thus, a comparative study of potassium contents in 14 hydroponically grown plant species (1 mmol/L K^+ in the nutritional solution) revealed potassium contents in the range of 153–274 mmol/kg fresh weight with a drop to 15–53 mmol/kg under a 1000-fold lower potassium supply (1 μmol/L) [90].

In most arable fields, potassium deficiency becomes a limiting factor for sustainable plant growth and development [91]. Therefore, the effects of potassium deficiency on crop plants are intensively studied under hydroponic conditions, which allow reliable defining precise potassium concentrations in nutrient solutions [92]. These experiments revealed the major visual symptoms of potassium deficiency as brown scorching and curling of leaf tips, as well as interveinal chlorosis caused by early chlorophyll degradation induced by ROS generation [92]. At the metabolic level, potassium deficit leads to the accumulation of carbohydrates (mainly sucrose) in leaves [8]. Most likely, this disaccharide plays the role of an osmoprotector, maintaining cell turgor under stressed conditions in plants [6], although some researchers attribute this effect to the enhancement of sucrose export from K^+-deficient leaves [8]. Importantly, in contrast to the conditions of nitrogen deprivation, potassium starvation does not result in any increase of root biomass in terms of an acclimation response to the restriction of potassium supply.

3.2 Regulation of K^+ transport systems and mobilization of vacuolar K^+ pools in plant responses to potassium deficiency

As was unambiguously proven by various analytical techniques, potassium is unequally distributed between different cell compartments, strongly dominating in cytosol and vacuole [80]. Thereby, cytosol and vacuole act as the major depots of potassium in plant cells [93]. This fact is essential for understanding the role of the ion homeostasis system in the mechanisms, underlying cell responses to potassium deficiency. Thus, in barley, the concentrations (activities) of K^+ in cytosol of both root and leaf cells measured with triple-barrelled microelectrodes (recording K^+ activity, pH and membrane potential) typically lie in the range of 100–200 mmol/L [93, 94]. Other methods, like K^+ efflux analysis, X-ray microanalysis or application of fluorescent dyes (reviewed by Britto and Kronzucker [95]) showed a larger range of values (30–320 mmol/L) which was probably due to a strong variation in the supply of potassium in cited experiments. Importantly, in cytosol, K^+ cannot be replaced by other cations, for example, Na^+ [78], that is, it is a specific cytosolic cation. In contrast to the cytosolic pool, the concentration of vacuolar potassium can vary between 10 and 500 mmol/L (i.e. 50-fold), depending on the plant species, cell type and potassium availability in soil [2, 80]. For example, in most glycophytes, it is ranging from approximately 120 mmol/L in root cell vacuoles [93] to 230 mmol/L in the vacuoles of leaf mesophyll cells [94]. In contrast to the cytosol, vacuolar K^+ can be, at least to some extent, replaced by other osmotica (i.e. sucrose, Na^+ or Mg^{2+}) [70, 96]. Vacuolar potassium contents are in a good agreement with the K^+ concentrations in the apoplast, which vary between 10 and 200 mmol/L, sometimes reaching up to 500 mmol/L [83, 97].

The responses of vacuolar and cytosolic K^+ pools to potassium deficiency were intensively studied since the 1980s, when the depletion of vacuolar K^+, accompanied by the accumulation of replacing cations (Na^+ and Mg^{2+}) in the vacuole, was proposed to be the earliest response of the plant cell to potassium starvation [80]. Remarkably, these alterations were not accompanied with significant changes in cytosolic potassium levels, that is, K^+-dependent processes in cytosol remain mostly unaffected. Further, it was proposed that the vacuolar potassium pool can be depleted only to a certain minimal value (10–20 mmol/L) [80]. However, subsequent determination of potassium contents in barley, relying on the measurements with triple-barrelled microelectrodes and a 14-day exposure of plants to 2 µmol/L K^+ in nutrient solution, revealed quite different responses of the vacuolar and cytosolic potassium pools in two types of root cells [93]. On the one hand, potassium concentrations (expressed as activities) demonstrated a concerted

decrease in the vacuole and cytosol from 122 to 124 mmol/L to 10 and 18 mmol/L, respectively. On the other hand, cytosolic concentrations in general showed less of a decline, more pronounced, however, in epidermal (from 81 to 45 mmol/L) than the cortical cells (from 83 to only 67 mmol/L) [93, 98].

Thermodynamic calculations of the cellular potassium homeostasis, performed to explain this phenomenon in cells severely depleted in K^+, clearly indicated the existence of an active transport mechanism for the translocation of K^+ from the vacuole to the cytosol which might rely on a 1:1 H^+:K^+ symport [93]. One also needs to take into account that cortical cells might have a higher capacity for the activation of K^+ influx into the cytosol via the high-affinity K^+ transporter HKT1, which is mostly associated with root cortical cells [16]. This might result in a higher ability of cortical cells to maintain a suitable potassium cytosolic concentration under nutrient starvation conditions. On the other hand, the observed difference can be explained by higher potassium losses via outward-rectifying K^+ channels in epidermal cells [93].

It is well known that cytosolic potassium homeostasis relies on the activities of multiple transport mechanisms, localized in cellular and organelle membranes [75]. The sophisticated network of potassium transport systems, involved in potassium absorption by roots, transport to shoots and further allocation within organs and cells, is the result of millions years of evolution of terrestrial plants [84]. Multiple elements of the potassium transport network were identified as K^+ channels, transporters and their regulators, comprehensively characterized during the last two decades [77, 78, 84, 99–104].

Thus, in Arabidopsis, seven major families of K^+ channels and transporters, comprising in total 75 genes, are known [78, 84]. The most studied and well-characterized families of plant potassium transport systems are (i) Shaker K^+ channel family, represented by voltage-gated channels; (ii) the tandem-pore K^+ (TPK) channels; and (iii) high-affinity K^+/K^+ uptake/K^+ transporter (KUP/HAK/KT) family of high-affinity K^+ transporters [103]. Among the Shaker K^+ channels, Arabidopsis K^+ transporter 1 (AKT1), K^+ Arabidopsis transporters 1 and 2 (KAT1, KAT2) and *Arabidopsis thaliana* K^+ channel 1 (AtKC1) are K^+ inwardly rectifying (Kin) channels. They are activated by membrane hyperpolarization and mediate potassium uptake, whereas stellar K^+ outward-rectifying (SKOR) and guard cell outward-rectifying (GORK) K^+ channels form Kout channels activated by membrane depolarization and mediate potassium release [78, 99, 103]. Finally, *Arabidopsis* K^+ transporter AKT2/3 is a weakly rectifying channel, switching from an inwardly rectifying to a non-rectifying state, mediating both potassium uptake and release depending on the local potassium electrochemical gradient [99, 103]. The Shaker channels are present in all plant organs [102] and ubiquitously expressed in various tissues. Being the main contributors in the potassium membrane fluxes [100, 101], they give access to fast and fine adjustments in K^+ transport in plant cells and for the redistribution of K^+ between distinct plant sections and cellular compartments to match plant demands under challenging environmental conditions [84]. AKT1, expressed predominantly in root epidermal cells [88], has been identified in Arabidopsis as a key K^+ channel protein, involved in potassium uptake under low (nearly 10 μmol/L) soil K^+ concentration [105]. The AtKC1, expressed in root cortex, epidermis and root hairs [106], is thought to be rather a regulatory subunit that does not form an own functional channel but interacts with AKT1 to form a functional heterotetrameric channel, which prevents AKT1-mediated K^+ loss under potassium starvation conditions [107]. It was shown that SKOR mediates the transfer of potassium to xylem sap for further transport to shoots [78]. Analogously, AKT2 substantially contributes to the phloem K^+ loading and unloading for long-distance potassium transport from sources to sinks [78].

The biological roles of the plant TPK K^+-selective channels are much less understood than those of the Shakers [78]. The representatives of the TPK family differ by their intracellular localization, while TPK4 is targeted to the plasma membrane; the other family members—TPK1, 2, 3 and 5—are located in organelle membranes [108]. Among them, due to its high selectivity, TPK1 appears to be mostly involved in the response to K^+ deficiency [109]. This protein is ubiquitously expressed in the tonoplast of guard and mesophyll cells and seems to impact on the vacuolar release of K^+ and on intracellular K^+ homeostasis [110].

The high-affinity K^+ transporters from the KUP/HAK/KT family are directly involved in maintaining the constant influx of K^+ in plant roots under severe potassium deficiency [78, 102]. The best characterized transporters of this group are AtHAK5 from Arabidopsis and its homologs in other species, which are assumed to be involved in K^+ uptake from very dilute potassium soil solutions, in co-transport with protons [103, 111, 112]. Besides this, AtKUP1 is described as a dual-affinity transporter in the plasma membrane of root cells and assumed to be essential in K^+ uptake [113], whereas AtKUP 2, 4, 6 and 8 are supposed to mediate K^+ efflux in root cells [78]. Similarly to HAK5, its homologs in other plant species (rice, barley, pepper, tomato) are highly up-regulated by potassium starvation [103]. It is generally recognized that HAK5 and AKT1 are the two main players in K^+ uptake from the soils, characterized with potassium shortage [102, 114, 115]. Indeed, the studies with T-DNA insertion lines clearly demonstrated that AtHAK5 is the only system mediating K^+ uptake at external potassium concentrations below 10 μmol/L [116], whereas both AtHAK5 and AKT1 systems contribute to K^+ absorption, when potassium concentrations are in the range of 10–200 μmol/L [117]. In particular, the uptake of K^+ in AtHAK5 AKT1 double mutant plants under potassium starvation conditions was reduced by 85% in comparison to the wild-type plants [115].

KUPs are found in plasma membrane and organelle membranes, and, in addition to K^+ uptake from soil, they are involved in K^+ homeostasis, long-distance K^+ transport, cell elongation, response to osmotic stress and even in the regulation of auxin transport [111]. Recently AtKUP7 in Arabidopsis roots was shown to contribute to K^+ uptake and K^+ efflux to the xylem especially under limited access to potassium [118]. The schematic view of the mechanisms behind the plant responses to potassium deficiency is shown in **Figure 2**. Less studied are high-affinity K^+ and Na^+ transporters from the HKT family. These proteins are expressed exclusively in the tonoplast. However, K^+-transporting members of the family seem to be present only in monocots [104]. This family is poorly characterized with regard to post-translational regulation, although some of its representatives are definitely involved in the control of K^+ homeostasis [78, 119].

Recently, elucidation of the mechanisms underlying the regulation of potassium transport in response to potassium starvation stress became a new focus of research, especially those acting both on the transcription and post-transcription levels [77, 102]. Various signaling cascades, enhancing transport of K^+, triggered by potassium starvation, might rely on reactive oxygen species, phytohormones, calcium and phosphatidic acid [8, 77]. Among the mentioned regulatory pathways, involved in the response to potassium deficiency, due to its spatial and temporal specificity, calcium signaling seems to be the most important one [77, 120]. In response to K^+ deprivation, the intracellular calcium levels change that affects peptide calcium sensors CBL1 and CBL9, localized in the plasma membrane (PM) [102]. The affected peptides interact with the cytoplasm-localized protein kinase CIPK23, and the formed complex is recruited to the root cell plasma membrane where it activates the AKT1 channel protein via phosphorylation [77, 121, 122] that results in enhancement of AKT1-mediated root uptake of K^+. Besides, AtKC1 as a channel regulatory subunit interacts with AtAKT1, forming an AtAKT1-AtKC1

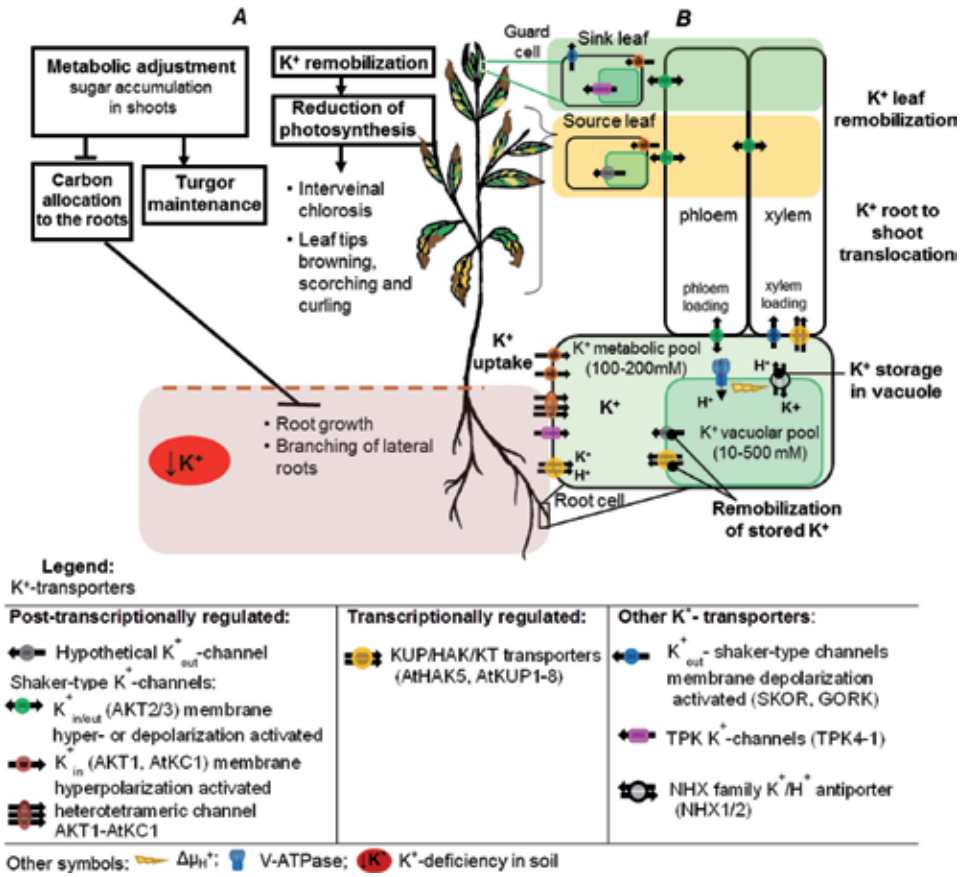

Figure 2.
Schematic view of plant responses to potassium deficiency. (A) The effect of K^+ deficiency on carbon metabolism in plants. K^+ deficiency in soil triggers metabolic adjustment in plant tissues to maintain cell turgor. However, in contrast to the NO_3^- deficiency response, this adjustment does not affect the carbon flow from shoots to roots and therefore does not influence root growth and branching. In source leaves, remobilization of K^+ results in suppression of photosynthesis, inter-veinal chlorosis and tip browning of leaves. Scorching and curling serve as the most prominent indicators of K^+ deficiency. (B) The major transport systems, involved in plant response to K^+ deficiency, that is, contributing in K^+ uptake, accumulation and remobilization in root cell, as well as distribution between root cells, translocation from root to shoot and from source to sink leaves, control of stomata guard cells.

heterotetrameric channel [123]. The regulatory subunit modulates the activity of AtAKT1 together with AtCIPK23 in a synergistic way, coordinating AtAKT1-mediated low-potassium stress responses [123, 124]. Another proposed mechanism relies on the calcium sensor CBL4, acting together with the protein kinase CIPK6, modulating activity and PM localization of the weak inward-rectifying K^+ channel AKT2 in Arabidopsis [125]. Thereby, CBL4 mediates translocation from the endoplasmic reticulum membrane to PM and enhances AKT2 activity.

There are indications that the calcium sensors CBL3 and CIPK9 work together and impact on K^+ homeostasis under low (100 μmol/L) potassium nutritional stress via the regulation of putative outward K^+ channels, localized in tonoplast [97]. However, other authors assume that CIPK9 is more likely involved in K^+ reallocation from older to the younger leaves under the conditions of potassium deficiency [126, 127]. It was also proposed that the AtCBL1/AtCIPK23 complex can phosphorylate the AtHAK5 transporter, which belongs to the KUP/HAK/KT family and expressed mainly in roots [128]. However, in contrast to AKT1, transcriptional regulation of the K^+ transporters seems to be more important for the adaptive response to potassium deficiency than

post-translational modification of K^+ channels [102, 129, 130]. Potassium starvation was shown to increase the abundance of HAK transcripts in a wide variety of plants, including barley, rice, *Arabidopsis thaliana*, *Solanum lycopersicum* and some others [19]. Thereby, the mRNA levels of HAK1-type genes were most remarkably increased [17, 105, 129]. The mechanisms, underlying expressional regulation of the HAK1-type genes might rely on alterations of membrane potential, as well as to reactive oxygen species (ROS) and to hormone-mediated signaling [105]. Recently, it was shown that transcription of AtHAK5 in *A. thaliana* roots can be induced by low-potassium nutritional stress via the transcription factor RAP2.11 that directly binds to the promoter region of *AtHAK5* and may be involved in the low-potassium signaling pathway [88]. The HAK/KUP/KT transporters were proposed to act as K^+-H^+ symporters in the tonoplast and might mobilize K^+ from the vacuole under potassium deficiency conditions [131]. Indeed, in *Arabidopsis thaliana*, OsHAK10 and five members of the KUP family have been found to be localized in the tonoplast [19] that supports the above functional assumption.

Thus, numerous studies in the field of K^+ membrane transport and intracellular potassium distribution in plants with regard to the changes in the availability of potassium in the environment indicate that the mechanisms supporting ion homeostasis of the plant cell might be involved in plant responses to potassium deficiency to ensure stress adaptation.

4. Conclusion

Nutrient deficiency, including moderate or severe shortages of NO_3^- and K^+ in soils, represents a serious challenge to modern agriculture, negatively affecting plant productivity and crop yields. Fortunately, plants possess an array of finely tuned mechanisms of nutritional stress adjustment and maintenance of cell ion homeostasis. Hence, the plant response to nitrate and potassium deficiency relies both on transcriptional and post-transcriptional regulation of high-affinity NO_3^- and K^+ membrane transport mechanisms, impacting on the increase of abundance and activity of transporters. Importantly, this includes not only an increase in the activity of root ion carriers but also mobilization of NO_3^- and K^+ from their storage vacuolar pools and subsequent redistribution to the metabolic cytosolic pool. In general, these data indicate that the ion homeostasis system plays an important role in plant cell responses to nutrient deficiency. Since these responses depend on plant taxonomy and duration of K^+ or NO_3^- shortage, these studies need to be extended to a broad selection of crop plants. To characterize the adaptive potential of these plants, various exposure times need to be addressed.

Currently, proteomics and metabolomics studies, aiming to improve stress tolerance in crop plants, became mainstream in the study of K^+ or NO_3^- starvation. The use of these techniques in research on nutrient stresses seems to be promising. Indeed, these approaches deliver valuable information about the accumulation of important secondary metabolites in plants under different types of environmental stresses.

Acknowledgements

This work was supported by the Russian Science Foundation (project number 17-16-01042) and the EU through EFRE with the state of Saxony-Anhalt (Agrochemical Institute Piesteritz, IB grant no. ZS/2016/04/78153).

Abbreviations

AKT	Arabidopsis K^+ transporter
AtAKT	*Arabidopsis thaliana* Arabidopsis K^+ transporter
AtCLCa	*Arabidopsis thaliana* chloride channel a
AtHAK	*Arabidopsis thaliana* high-affinity K^+ transporter family
AtKC	*Arabidopsis thaliana* K^+ channel
AtKUP	*Arabidopsis thaliana* K^+ uptake transporter family
CBL	calcineurin B-like
CIPK	calcineurin B-like-interacting protein kinase
CLC	chloride channel family
GORK	guard cell outward-rectifying K^+ channel
HAK/KUP/KT	high-affinity K^+/K^+ uptake/K^+ transporter family
HKT	high-affinity K^+ transporter
K	potassium
KAT	K^+ Arabidopsis transporter
K_m	Michaelis-Menten constant
N	nitrogen
NAR	nitrate transporter-activating protein
NO_3^-	nitrate
NR	nitrate reductase
NRT	nitrate transporter
NRT/NPF	nitrate transporter/peptide transporter
OsHAK	*Oryza sativa* high-affinity K^+ transporter family
P	phosphorous
PM	plasma membrane
RNA	ribonucleic acid
ROS	reactive oxygen species
SKOR	stelar K^+ outward-rectifying K^+ channels
SLAC/SLAH	slow anion channel-associated homologs
T-DNA	transfer deoxyribonucleic acid
TPK	tandem-pore K^+ channel
V-ATPase	the vacuolar H^+-translocating adenosine triphosphatase
V-PPase	the vacuolar H^+-pumping pyrophosphatase

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References

[1] Lopez-Arredondo DL, Leyva-Gonzalez MA, Alatorre-Cobos F, Herrera-Estrella L. Biotechnology of nutrient uptake and assimilation in plants. The International Journal of Developmental Biology. 2013;**57**: 595-610. DOI: 10.1387/ijdb.130268lh

[2] Marschner H. Mineral Nutrition of Higher Plants. 2nd ed. London: Academic Press; 1995. 889 p. DOI: 10.1016/B978-0-12-473542-2.X5000-7

[3] Kulcheski FR, Correa R, Gomes IA, de Lima JC, Margis R. NPK macronutrients and micro RNA homeostasis. Frontiers in Plant Science. 2015;**6**:451. DOI: 10.3389/fpls.2015.00451

[4] Tan ZX, Lal R, Wiebe KD. Global soil nutrient depletion and yield reduction. Journal of Sustainable Agriculture. 2005;**26**:123-146. DOI: 10.1300/J064v26n01_10

[5] Pessarakli M, Haghighi M, Sheibanirad A. Plant responses under environmental stress conditions. Advances in Plants & Agriculture Research. 2015;**2**:276-286. DOI: 10.15406/apar.2015.02.00073

[6] Cakmak I, Hengeler C, Marschner H. Changes in phloem export of sucrose in leaves in response to phosphorus, potassium and magnesium deficiency in bean plants. Journal of Experimental Botany. 1994;**45**:1251-1257. DOI: 10.1093/jxb/45.9.1251

[7] Hirai MY, Yano M, Goodenowe DB, Kanaya S, Kimura T, Awazuhara M, et al. Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in Arabidopsis thaliana. Proceedings of the National Academy of Sciences. 2004;**101**:10205-10210. DOI: 10.1073/pnas.0403218101

[8] Hermans C, Hammond JP, White PJ, Verbruggen N. How do plants respond to nutrient shortage by biomass allocation? Trends in Plant Science. 2006;**11**:610-617. DOI: 10.1016/j.tplants.2006.10.007

[9] Wang R, Okamoto M, Xing X, Crawford NM. Microarray analysis of the nitrate response in Arabidopsis root sands hoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiology. 2003;**32**:556-567. DOI: 10.1104/pp.103.021253

[10] Wu P, Ma L, Hou X, Wang M, Wu Y, Liu F, et al. Phosphate starvation triggers distinct alterations of genome expression in Arabidopsis roots and leaves. Plant Physiology. 2003;**132**:1260-1271. DOI: 10.1104/pp.103.021022

[11] Maathuis FJ, Filatov V, Herzyk P, Krijger GC, Axelsen KB, Chen S, et al. Transcriptome analysis of root transporters reveals participation of multiple gene families in the response to cation stress. The Plant Journal. 2003;**35**:675-692. DOI: 10.1046/j.1365-313X.2003.01839.x

[12] Price J, Laxmi A, Martin SK, Jang JC. Global transcription profiling reveals multiple sugar signal transduction mechanisms in Arabidopsis. The Plant Cell. 2004;**16**:2128-2150. DOI: 10.1105/tpc.104.022616

[13] Scheible WR, Morcuende R, Czechowski T, Fritz C, Osuna D, Palacios-Rojas N, et al. Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of Arabidopsis in response to nitrogen. Plant Physiology. 2004;**136**:2483-2499. DOI: 10.1104/pp.104.047019

[14] Abel S. Phosphate sensing in root development. Current Opinion in Plant Biology. 2011;**14**:303-309. DOI: 10.1016/j.pbi.2011.04.007

[15] Abel S. Phosphate scouting by root tips. Current Opinion in Plant Biology. 2017;**39**:168-177. DOI: 10.1016/j.pbi.2017.04.016

[16] Schachtman DP, Schroeder JI. Structure and transport mechanism of a high-affinity K^+ uptake transporter from higher plants. Nature. 1994;**370**:655-658. DOI: 10.1038/370655a0

[17] Wang YH, Garvin DF, Kochian LV. Rapid induction of regulatory and transporter genes in response to phosphorus, potassium, and iron deficiencies in tomato roots. Evidence for crosstalk and root/rhizosphere-mediated signals. Plant Physiology. 2002;**130**: 1361-1370. DOI: 10.1104/pp.008854

[18] Banuelos MA, Garciadeblas B, Cubero B, Rodrıguez-Navarro A. Inventory and functional characterization of the HAK potassium transporters of rice. Plant Physiology. 2002;**130**:784-795. DOI: 10.1104/pp.007781

[19] Szczerba MW, Britto DT, Kronzucker HJ. K^+ transport in plants: Physiology and molecular biology. Journal of Plant Physiology. 2009;**166**:447-466. DOI: 10.1016/j.jplph.2008.12.009

[20] Curci PL, Cigliano RA, Zuluaga DL, Janni M, Sanseverino W, Sonnante G. Transcriptomic response of durum wheat to nitrogen starvation. Scientific Reports. 2017;**7**:1176-1190. DOI: 10.1038/s41598-017-01377-0

[21] Wang J, Song K, Sun L, Qin Q, Sun Y, Pan J, et al. Morphological and transcriptome analysis of wheat seedlings response to low nitrogen stress. Plants. 2019;**8**:98. DOI: 10.3390/plants8040098

[22] Gelli M, Duo Y, Konda A, Zhang C, Holding D, Dweikat I. Identification of differentially expressed genes between sorghum genotypes with contrasting nitrogen stress tolerance by genome-wide transcriptional profiling. BMC Genomics. 2014;**15**:179. DOI: 10.1186/1471-2164-15-179

[23] Zhao X, Huang J, Yu H, Wang L, Xie W. Genomic survey, characterization and expression profile analysis of the peptide transporter family in rice (*Oryza sativa* L.). BMC Plant Biology. 2010;**10**:92. DOI: 10.1186/1471-2229-10-92

[24] Cai H, Lu Y, Xie W, Zhu T, Lian X. Transcriptome response to nitrogen starvation in rice. Journal of Biosciences. 2012;**37**:731-747. DOI: 10.1007/s12038-012-9242-2

[25] Martinoia E, Maeshima M, Neuhaus HE. Vacuolar transporters and their essential role in plant metabolism. Journal of Experimental Botany. 2007;**58**:83-102. DOI: 10.1093/jxb/erl183

[26] Liu Y, von Wiren N. Ammonium as a signal for physiological and morphological responses in plants. Journal of Experimental Botany. 2017;**68**(10): 2581-2592. DOI: 10.1093/jxb/erx086

[27] von Wiren N, Gazzarrini S, Gojon A, Frommer WB. The molecular physiology of ammonium uptake and retrieval. Current Opinion in Plant Biology. 2000;**3**(3):254-261

[28] De Angeli A, Thomine S, Frachisse JM, Ephritikhine G, Gambale F, Barbier-Brygoo H. Anion channels and transporters in plant cell membranes. FEBS Letters. 2007;**581**:2367-2374. DOI: 10.1016/j.febslet.2007.04.003

[29] Xu G, Fan X, Miller AJ. Plant nitrogen assimilation and use efficiency. Annual Review of Plant Biology. 2012;**63**:153-182. DOI: 10.1146/annurev-arplant-042811-105532

[30] Zarabi M, Jalali M. Leaching of nitrogen from calcareous soils in western Iran: A soil leaching column study. Environmental Monitoring and Assessment. 2012;**184**:7607-7622. DOI: 10.1007/s10661-012-2522-3

[31] Fan X, Naz M, Fan X, Xuan W, Miller AJ, Xu G. Plant nitrate transporters: From gene function to application. Journal of Experimental Botany. 2017;**68**:2463-2475. DOI: 10.1093/jxb/erx011

[32] Miller AJ, Fan X, Orsel M, Smith SJ, Wells DM. Nitrate transport and signalling. Journal of Experimental Botany. 2007;**58**:2297-2306. DOI: 10.1093/jxb/erm066

[33] Miller AJ, Smith SJ. Cytosolic nitrate ion homeostasis: Could it have a role in sensing nitrogen status? Annals of Botany. 2008;**101**:485-489. DOI: 10.1093/aob/mcm313

[34] Crawford NM. Nitrate: Nutrient and signal for plant growth. The Plant Cell. 1995;**7**:859-868. DOI: 10.1105/tpc.7.7.859

[35] Stitt M. Nitrate and the regulation of primary metabolism, allocation and growth. Current Opinion in Plant Science. 1999;**2**:178-186. DOI: 10.1016/S1369-5266(99)80033-8

[36] Makino A, Osmond B. Effects of nitrogen nutrition on nitrogen partitioning between chloroplast and mitochondria in pea and wheat. Plant Physiology. 1991;**96**:355-362. DOI: 10.1104/pp.96.2.355

[37] Zhao L, Zhang W, Yang Y, Li Z, Li N, Qi S, et al. The Arabidopsis NLP7 gene regulates nitrate signaling via NRT1.1-dependent pathway in the presence of ammonium. Scientific Reports. 2018;**8**:1487. DOI: 10.1038/s41598-018-20038-4

[38] Miller AJ, Cramer MD. Root nitrogen acquisition and assimilation. Plant and Soil. 2004;**274**:1-36. DOI: 10.1007/s11104-004-0965-1

[39] Giehl RF, Gruber BD, von Wiren N. It's time to make changes: Modulation of root system architecture by nutrient signals. Journal of Experimental Botany. 2013;**65**(3):769-778. DOI: 10.1093/jxb/ert421

[40] Kiba T, Krapp A. Plant nitrogen acquisition under low availability: Regulation of uptake and root architecture. Plant & Cell Physiology. 2016;**57**:707-714. DOI: 10.1093/pcp/pcw052

[41] O'Brien JA, Vega A, Bouguyon E, Krouk G, Gojon A, Coruzzi G, et al. Nitrate transport, sensing, and responses in plants. Molecular Plant. 2016;**9**:837-856. DOI: 10.1016/j.molp.2016.05.004

[42] Salt DE. Update on plant ionomics. Plant Physiology. 2004;**136**:2451-2456. DOI: 10.1104/pp.104.047753

[43] Huang XY, Salt DE. Plant ionomics: From elemental profiling to environmental adaptation. Molecular Plant. 2016;**9**:787-797. DOI: doi.org/10.1016/j.molp.2016.05.003

[44] Coruzzi G, Bush DR. Nitrogen and carbon nutrient and metabolite signalling in plants. Plant Physiology. 2001;**125**:61-64. DOI: 10.1104/pp.125.1.61

[45] Hirel B, Le Gouis J, Ney B, Gallais A. The challenge of improving nitrogen use efficiency in crop plants: Towards a more central role for genetic variability and quantitative genetics within integrated approaches. Journal of Experimental Botany. 2007;**58**:2369-2387. DOI: 10.1093/jxb/erm097

[46] Krapp A, David LC, Chardin C, Girin T, Marmagne A, Leprince AS, et al. Nitrate transport and signalling in Arabidopsis. Journal of Experimental

Botany. 2014;**65**:789-798. DOI: 10.1093/jxb/eru001

[47] Wang YY, Hsu PK, Tsay YF. Uptake, allocation and signaling of nitrate. Trends in Plant Science. 2012;**17**:458-467. DOI: 10.1016/j.tplants.2012.04.006

[48] Liu KH, Tsay YF. Switching between the two action modes of the dual-affinity nitrate transporter CHL1 by phosphorylation. The EMBO Journal. 2003;**22**(5):1005-1013. DOI: 10.1093/emboj/cdg118

[49] Gojon A, Krouk G, Perrine-Walker F, Laugier E. Nitrate transceptor(s) in plants. Journal of Experimental Botany. 2011;**62**: 2299-2308. DOI: 10.1093/jxb/erq419

[50] Dechorgnat J, Nguyen CT, Armengaud P, Jossier M, Diatloff E, Filleur S, et al. From the soil to the seeds: The long journey of nitrate in plants. Journal of Experimental Botany. 2011;**62**:1349-1359. DOI: 10.1093/jxb/erq409

[51] Lezhneva L, Kiba T, Feria-Bourrellier AB, Lafouge F, Boutet-Mercey S, Zoufan P, et al. The Arabidopsis nitrate transporter NRT2.5 plays a role in nitrate acquisition and remobilization in nitrogen-starved plants. Plant Journal. 2014;**80**:230-241. DOI: 10.1111/tpj.12626

[52] Zhang ZH, Huang HT, Song HX, Liu Q, Rong XM, Peng JW, et al. Research advances on nitrate nitrogen reutilization by proton pump of tonoplast and its relation to nitrogen use efficiency. Australian Journal of Crop Science. 2012;**6**:1377-1382

[53] Ferrari TE, Yoder OC, Filner P. Anaerobic nitrite production by plant cells and tissues: Evidence for two nitrate pools. Plant Physiology. 1973;**51**:423-431. DOI: 10.1104/pp.51.3.423

[54] Martinoia E, Heck U, Wiemken A. Vacuoles as storage compartments of nitrate in barley leaves. Nature. 1981;**289**:292-294. DOI: 10.1038/289292a0

[55] Siddiqi MY, Glass ADM. An evaluation of the evidence for, and implications of, cytoplasmic nitrate homeostasis. Plant, Cell and Environment. 2002;**25**:1211-1217. DOI: 10.1046/j.1365-3040.2002.00927.x

[56] Britto DT, Kronzucker HJ. The case for cytosolic NO_3 heterostasis: A critique of a recently proposed model. Plant, Cell and Environment. 2003;**26**:83-188. DOI: 10.1046/j.1365-3040.2003.00992.x

[57] De Angeli A, Monachello D, Ephritikhine G, Frachisse JM, Thomine S, Gambale F, et al. The nitrate/proton antiporter AtCLCa mediates nitrate accumulation in plant vacuoles. Nature. 2006;**442**:939-942. DOI: 10.1038/nature05013

[58] Granstedt RC, Huffaker RC. Identification of the leaf vacuole as a major nitrate storage pool. Plant Physiology. 1982;**70**:410-413. DOI: 10.1104/pp.70.2.410

[59] Martinoia E, Massoneau A, Frangne N. Transport processes of solutes across the vacuolar membrane of higher plants. Plant and Cell Physiology. 2000;**41**:1175-1181. DOI: 10.1093/pcp/pcd059

[60] Fan XR, Jia LJ, Li YL, Smith SJ, Miller AJ, Shen QR. Comparing nitrate storage and remobilization in two rice cultivars that differ in their nitrogen use efficiency. Journal of Experimental Botany. 2007;**58**:1729-1740. DOI: 10.1093/jxb/erm033

[61] Cookson SJ, Williams LE, Miller AJ. Light-dark changes in the cytosolic nitrate pools depend on nitrate reductase activity in Arabidopsis leaf

cells. Plant Physiology. 2006;**138**: 1097-1105. DOI: 10.1104/pp.105.062349

[62] Macduf JH, Jarvis SC, Masquera A. Nitrate nutrition of grasses from steady-state supplies in flowing solution culture following nitrate deprivation and/or defoliation. II. Assimilation of NO_3^- and short-term effects on NO_3^- uptake. Journal of Experimental Botany. 1989;**40**:977-984. DOI: 10.1093/jxb/40.9.977

[63] Van der Leij M, Smith SJ, Miller AJ. Remobilization of vacuole stored nitrate in barley root cells. Planta. 1998;**205**: 64-72. DOI: 10.1007/s004250050297

[64] Lea US, ten Hoopen F, Provan F, Kaiser WM, Meyer C, Lillo C. Mutation of the regulatory phosphorylation site of tobacco nitrate reductase results in high nitrite excretion and NO emission from leaf and roots tissue. Planta. 2004;**219**:59-65. DOI: 10.1007/s00425-004-1209-6

[65] Han YL, Song HX, Liao Q, Yu Y, Jian SF, Lepo JE, et al. Nitrogen use efficiency is mediated by vacuolar nitrate sequestration capacity in roots of Brassica napus. Plant Physiology. 2016;**170**: 1684-1698. DOI: 10.1104/pp.15.01377

[66] Glass ADM, Britto DT, Kaiser BN, Kinghorn JR, Kronzucker HJ, Kumar A, et al. The regulation of nitrate and ammonium transport systems in plants. Journal of Experimental Botany. 2002;**53**:855-864. DOI: 10.1093/jexbot/53.370.855

[67] He YN, Peng JS, Cai Y, Liu DF, Guan Y, Yi HY, et al. Tonoplast-localized nitrate uptake transporters involved in vacuolar nitrate efflux and reallocation in Arabidopsis. Scientific Reports. 2017;**7**:6417. DOI: 10.1038/s41598-017-06744-5

[68] Miller AJ, Smith SJ. Nitrate transport and compartmentation in cereal root cells. Journal of Experimental Botany. 1996;**47**:843-854. DOI: 10.1093/jxb/47.7.843

[69] Blumwald E, Poole RJ. Nitrate storage and retrieval in Beta vulgaris: Effects of nitrate and chloride on proton gradients in tonoplast vesicles. Proceedings of the National Academy of Sciences. 1985;**82**:3683-3687. DOI: 10.1073/pnas.82.11.3683

[70] Veen BW, Kleinendorst A, Lambers H, Neeteson JJ, Stulen I. The role of nitrate in osmoregulation of Italian ryegrass. Fundamental, Ecological and Agricultural Aspects of Nitrogen Metabolism in Higher Plants. Developments in Plant and Soil Sciences. 1986;**19**:477-480. DOI: 10.1007/978-94-009-4356-8_70

[71] Osmolovskaya NG, Kuchaeva LN, Dung VV, Popova NF. Hydroponic technologies in the management of yield and the quality of plant production. Agrophysika. 2017;**2**:19-29

[72] Tegeder M, Masclaux-Daubresse C. Source and sink mechanisms of nitrogen transport and use. The New Phytologist. 2018;**217**:35-53. DOI: 10.1111/nph.14876

[73] Fan SC, Lin CS, Hsu PK, Lin SH, Tsay YF. The Arabidopsis nitrate transporter NRT1.7, expressed in phloem, is responsible for source-to-sink remobilization of nitrate. The Plant Cell. 2009;**21**:2750-2761. DOI: 10.1105/tpc.109.067603

[74] Okamoto M, Vidmar JJ, Glass ADM. Regulation of NRT1 and NRT2 gene families of *Arabidopsis thaliana*: Responses to nitrate provision. Plant and Cell Physiology. 2003;**44**:304-317. DOI: 10.1093/pcp/pcg036

[75] Kiba T, Feria-Bourrellier AB, Lafouge F, Lezhneva L, Boutet-Mercey S, Orsel M, et al. The Arabidopsis nitrate transporter NRT2.4 plays a double role

in roots and shoots of nitrogen starved plants. The Plant Cell. 2012;**24**:245-258. DOI: 10.1105/tpc.111.092221

[76] Hsu PK, Tsay YF. Two phloem nitrate transporters, NRT1.11 and NRT1.12, are important for redistributing xylem-borne nitrate to enhance plant growth. Plant Physiology. 2013;**163**:844-856. DOI: 10.1104/pp.113.226563

[77] Wang X, Hao L, Zhu B, Jiang Z. Plant calcium signaling in response to potassium deficiency. International Journal of Molecular Sciences. 2018;**19**:3456. DOI: 10.3390/ijms19113456

[78] Cherel I, Gaillard I. The complex fine-tuning of K^+ fluxes in plants in relation to osmotic and ionic abiotic stresses. International Journal of Molecular Sciences. 2019;**20**:715. DOI: 10.3390/ijms20030715

[79] Amtmann A, Blatt MR. Regulation of macronutrient transport. The New Phytologist. 2009;**181**:35-52. DOI: 10.1111/j.1469-8137.2008.02666.x

[80] Leigh RA, Wyn Jones RG. A hypothesis relating critical potassium concentrations for growth to the distribution and functions of this ion in the plant cell. The New Phytologist. 1984;**97**:1-13. DOI: 10.1111/j.1469-8137.1984.tb04103.x

[81] Amtmann A, Troufflard S, Armengaud P. The effect of potassium nutrition on pest and disease resistance in plants. Physiologia Plantarum. 2008;**133**:682-691. DOI: 10.1111/j.1399-3054.2008.01075.x

[82] Maathuis FJ. Physiological functions of mineral macronutrients. Current Opinion in Plant Biology. 2009;**12**: 250-258. DOI: 10.1016/j.pbi.2009.04.003

[83] Wang M, Zheng Q, Shen Q, Guo S. The critical role of potassium in plant stress response. International Journal of Molecular Sciences. 2013;**14**:7370-7390. DOI: 10.3390/ijms14047370

[84] Shabala S, Pottosin II, Demidchik V, Maathuis F. Potassium and potassium-permeable channels in plant salt tolerance. In: Ion Channels and Plant Stress Responses. Signaling and Communication in Plants. Vol. 87. Berlin, Heidelberg: Springer; 2010. p. 110. DOI: 10.1007/978-3-642-10494-7_5

[85] Demidchik V. Mechanisms and physiological roles of K^+ efflux from root cells. Journal of Plant Physiology. 2014;**171**:696-707. DOI: 10.1016/j.jplph.2014.01.015

[86] Manning DAC. Mineral sources of potassium for plant nutrition. A review. Agronomy for Sustainable Development. 2010;**30**:281-294. DOI: 10.1051/agro/2009023

[87] Moody PW, Bell MJ. Availability of soil potassium and diagnostic soil tests. Australian Journal of Soil Research. 2006;**44**:265-275. DOI: 10.1071/SR05154

[88] Aleman F, Nieves-Cordones M, Martínez V, Rubio F. Root K^+ acquisition in plants: The *Arabidopsis thaliana* model. Plant & Cell Physiology. 2011;**52**:1603-1612. DOI: 10.1093/pcp/pcr096

[89] Jungk A, Claassen N. Availability of phosphate and potassium as the result of interactions between root and soil in the rhizosphere. Journal of Plant Nutrition and Soil Science. 1986;**149**:411-427. DOI: 10.1002/jpln.19861490406

[90] Asher CJ, Ozanne PG. Growth and potassium contents of plants in solution cultures maintained at constant potassium concentrations. Soil Science. 1967;**103**:155-161. DOI: 10.1097/00010694-196703000-00002

[91] Hafsi C, Falleh H, Saada M, Ksouri R, Abdelly C. Potassium deficiency alters growth, photosynthetic performance, secondary metabolites content, and related antioxidant capacity in sulla carnosa grown under moderate salinity. Plant Physiology and Biochemistry. 2017;**118**:609-617. DOI: 10.1016/j.plaphy.2017.08.002

[92] Du Q, Zhao XH, Xia L, Jiang C, Wang X, Han Y, et al. Effects of potassium deficiency on photosynthesis, chloroplast ultrastructure, ROS, and antioxidant activities in maize (*Zea mays* L.). Journal of Integrative Agriculture. 2019;**18**:395-406. DOI: 10.1016/S2095-3119(18)61953-7

[93] Walker DJ, Leigh RA, Miller AJ. Potassium homeostasis in vacuolate plant cells. Proceedings of the National Academy of Sciences. 1996;**93**:10510-10514. DOI: 10.1073/pnas.93.19.10510

[94] Cuin TA, Miller AJ, Laurie SA, Leigh RA. Potassium activities in cell compartments of salt-grown barley leaves. Journal of Experimental Botany. 2003;**54**:657-661. DOI: 10.1093/jxb/erg072

[95] Britto DT, Kronzucker HJ. Cellular mechanisms of potassium transport in plants. Physiologia Plantarum. 2008;**133**:637-650. DOI: 10.1111/j.1399-3054.2008.01067.x

[96] Benito B, Haro R, Amtmann A, Cuin TA, Dreyer I. The twins K^+ and Na^+ in plants. Journal of Plant Physiology. 2014;**171**:723-731. DOI: 10.1016/j.jplph.2013.10.014

[97] White PJ, Karley AJ. Potassium. In: Hell R, Mendel RR, editors. Cell Biology of Metals and Nutrients. Plant Cell Monographs. Berlin, Heidelberg: Springer; 2010. pp. 199-224. DOI: 10.1007/978-3-642-10613-2_9

[98] Walker DJ, Black CR, Miller AJ. The role of cytosolic potassium and pH in the growth of barley roots. Plant Physiology. 1998;**118**:957-964. DOI: 10.1104/pp.118.3.957

[99] Very AA, Sentenac H. Molecular mechanisms and regulation of K^+ transport in higher plants. Annual Review of Plant Biology. 2003;**54**:575-603. DOI: 10.1146/annurev.arplant.54.031902.134831

[100] Lebaudy A, Very AA, Sentenac H. K^+ channel activity in plants: Genes, regulations and functions. FEBS Letters. 2007;**581**:2357-2366. DOI: 10.1016/j.febslet.2007.03.058

[101] Hedrich R. Ion channels in plants. Physiological Reviews. 2012;**92**:1777-1811. DOI: 10.1152/physrev.00038.2011

[102] Liu LL, Ren HM, Chen LQ, Wang Y, Wu WH. A protein kinase, calcineurin B-like protein-interacting protein kinase 9, interacts with calcium sensor calcineurin B-like protein 3 and regulates potassium homeostasis under low-potassium stress in Arabidopsis. Plant Physiology. 2013;**161**:266-277. DOI: 10.1104/pp.112.206896

[103] Very AA, Nieves-Cordones M, Daly M, Khan I, Fizames C, Sentenac H. Molecular biology of K^+ transport across the plant cell membrane: What do we learn from comparison between plant species? Journal of Plant Physiology. 2014;**171**:748-769. DOI: 10.1016/j.jplph.2014.01.011

[104] Ward JM, Maser P, Schroeder JI. Plant ion channels: Gene families, physiology, and functional genomics analyses. Annual Review of Physiology. 2009;**71**:59-82. DOI: 10.1146/annurev.physiol.010908.163204

[105] Kim MJ, Ruzicka D, Shin R, Schachtman DP. The Arabidopsis AP2/ERF transcription factor RAP2.11 modulates plant response to low potassium conditions. Molecular Plant.

2012;**5**:1042-1057. DOI: 10.1093/mp/sss003

[106] Pilot G, Gaymard F, Mouline K, Cherel I, Sentenac H. Regulated expression of Arabidopsis Shaker K^+ channel genes involved in K^+ uptake and distribution in the plant. Plant Molecular Biology. 2003;**51**:773-787. DOI: 10.1023/A:1022597102282

[107] Geiger D, Becker D, Vosloh D, Gambale F, Palme K, Rehers M, et al. Heteromeric AtKC1/AKT1 channels in Arabidopsis roots facilitate growth under K^+-limiting conditions. The Journal of Biological Chemistry. 2009;**284**:21288-21295. DOI: 10.1074/jbc.M109.017574

[108] Voelker C, Gomez-Porras JL, Becker D, Hamamoto S, Uozumi N, Gambale F, et al. Roles of tandem-pore K^+ channels in plants—A puzzle still to be solved. Plant Biology. 2010;**12**:56-63. DOI: 10.1111/j.1438-8677.2010.00353.x

[109] Bihler H, Eing C, Hebeisen S, Roller A, Czempinski K, Bertl A. TPK1 is a vacuolar ion channel different from the slow-vacuolar cation channel. Plant Physiology. 2005;**139**:417-424. DOI: 10.1104/pp.105.065599

[110] Gobert A, Isayenkov S, Voelker C, Czempinski K, Maathuis FJ. The two-pore channel TPK1 geneencodes the vacuolar K^+ conductance and plays a role in K^+ homeostasis. Proceedings of the National Academy of Sciences. 2007;**104**:10726-10731. DOI: 10.1073/pnas.0702595104

[111] Santa-Maria GE, Oliferuk S, Moriconi JI. KT-HAK-KUP transporters in major terrestrial photosynthetic organisms: A twenty years tale. Journal of Plant Physiology. 2018;**226**:77-90. DOI: 10.1016/j.jplph.2018.04.008

[112] Li W, Xu G, Alli A, Yu L. Plant HAK/KUP/KT K^+ transporters: Function and regulation. Seminars in Cell & Developmental Biology. 2018;**74**:133-141. DOI: 10.1016/j.semcdb.2017.07.009

[113] Fu HH, Luan S. AtKuP1: A dual-affinity K^+ transporter from Arabidopsis. The Plant Cell. 1998;**10**:63-73. DOI: 10.1105/tpc.10.1.63

[114] Rubio F, Nieves-Cordones M, Aleman F, Martinez V. Relative contribution of AtHAK5 and AtAKT1to K^+ uptake in the high-affinity range of concentrations. Physiologia Plantarum. 2008;**134**:598-608. DOI: 10.1111/j.1399-3054.2008.01168.x

[115] Pyo YJ, Gierth M, Schroeder JI, Cho MH. High-affinity K^+ transport in Arabidopsis: AtHAK5 and AKT1 are vital for seedling establishment and post germination growth under low-potassium conditions. Plant Physiology. 2010;**153**:863-875. DOI: 10.1104/pp.110.154369

[116] Rubio F, Arevalo L, Caballero F, Botella MA, Rubio JS, Garcıa-Sanchez F, et al. Systems involved in K^+ uptake from diluted solutions in pepper plants as revealed by the use of specific inhibitors. Journal of Plant Physiology. 2010;**167**:1494-1499. DOI: 10.1016/j.jplph.2010.05.022

[117] Rubio F, Aleman F, Nieves-Cordones M, Vicente M. Studies on Arabidopsis athak5, atakt1 double mutants disclose the range of concentrations at which AtHAK5, AtAKT1 and unknown systems mediate K^+ uptake. Physiologia Plantarum. 2010;**139**:220-228. DOI: 10.1111/j.1399-3054.2010.01354.x

[118] Han M, Wu W, Wu WH, Wang Y. Potassium transporter KUP7 is involved in K^+ acquisition and translocation in Arabidopsis root under K^+-limited conditions. Molecular Plant. 2016;**9**:437-446. DOI: 10.1016/j.molp.2016.01.012

[119] Sze H, Chanroj S. Plant endomembrane dynamics: Studies of K^+/H^+ antiporters provide insights on the effects of pH and ion homeostasis. Plant Physiology. 2018;**177**:875-895. DOI: 10.1104/pp.18.00142

[120] Behera S, Long Y, Schmitz-Thom I, Wang XP, Zhang C, Li H, et al. Two spatially and temporally distinct Ca^{2+} signals convey Arabidopsis thaliana responses to K^+ deficiency. The New Phytologist. 2017;**213**:739-750. DOI: 10.1111/nph.14145

[121] Xu J, Li HD, Chen LQ, Wang Y, Liu LL, He L, et al. A protein kinase, interacting with two calcineurin B-like proteins, regulates K^+ transporter AKT1 in Arabidopsis. Cell. 2006;**125**:1347-1360. DOI: 10.1016/j.cell.2006.06.011

[122] Li L, Kim BG, Cheong YH, Pandey GK, Luan S. A Ca^{2+} signaling pathway regulates a K^+ channel for low-K response in Arabidopsis. Proceedings of the National Academy of Sciences. 2006;**103**:12625-12630. DOI: 10.1073/pnas.0605129103

[123] Jeanguenin L, Alcon C, Duby G, Boeglin M, Cherel I, Gaillard I, et al. AtKC1 is a general modulator of Arabidopsis inward Shaker channel activity. The Plant Journal. 2011;**67**:570-582. DOI: 10.1111/j.1365-313X.2011.04617.x

[124] Wang XP, Chen LM, Liu WX, Shen LK, Wang FL, Zhou Y, et al. AtKC1 and CIPK23 synergistically modulate AKT1-mediated low-potassium stress responses in Arabidopsis. Plant Physiology. 2016;**170**:2264-2277. DOI: 10.1104/pp.15.01493

[125] Held K, Pascaud F, Eckert C, Gajdanowicz P, Hashimoto K, Corratge-Faillie C, et al. Calcium-dependent modulation and plasma membrane targeting of the AKT2 potassium channel by the CBL4/CIPK6 calcium sensor/protein kinase complex. Cell Research. 2011;**21**:1116-1130. DOI: 10.1038/cr.2011.50

[126] Pandey GK, Cheong YH, Kim BG, Grant JJ, Li L, Luan S. CIPK9: A calcium sensor-interacting protein kinase required for low-potassium tolerance in Arabidopsis. Cell Research. 2007;**17**:411-421. DOI: 10.1038/cr.2007.39

[127] Adams E, Shin R. Transport, signaling, and homeostasis of potassium and sodium in plants. Journal of Integrative Plant Biology. 2014;**56**:231-249. DOI: 10.1111/jipb.12159

[128] Ragel P, Rodenas R, Garcia-Martin E, Andres Z, Villalta I, Nieves-Cordones M, et al. The CBL-interacting protein kinase CIPK23 regulates HAK5-mediated high-affinity K^+ uptake in Arabidopsis roots. Plant Physiology. 2015;**169**:2863-2873. DOI: 10.1104/pp.15.01401

[129] Amtmann A, Hammond JP, Armengaud P, White PJ. Nutrient sensing and signalling in plants: Potassium and phosphorus. Advances in Botanical Research. 2005;**43**:209-257. DOI: 10.1016/S0065-2296(05)43005-0

[130] Gierth M, Maser P, Schroeder JI. The potassium transporter AtHAK5 functions in K^+ deprivation-induced high-affinity K^+ uptake and AKT1 K^+ channel contribution to K^+ uptake kinetics in Arabidopsis roots. Plant Physiology. 2005;**137**:1105-1114. DOI: 10.1104/pp.104.057216

[131] Rodriguez-Navarro A, Rubio F. High-affinity potassium and sodium transport systems in plants. Journal of Experimental Botany. 2006;**57**:1149-1160. DOI: 10.1093/jxb/erj068

Chapter 6

Intracellular Lipid Homeostasis and Trafficking in Autophagy

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Abstract

In eukaryotes, lipids are not only an important constituent of the plasma membrane but also used to generate specialized membrane-bound organelles, including temporary compartments with critical functions. As such, lipids play a key role in intracellular homeostasis—the ability of a cell to maintain stable internal conditions upon changes in its extracellular environment. Autophagy, one of the cellular processes through which eukaryotic cells strive for survival under stress, is heavily dependent on lipid and membrane trafficking through the *de novo* formation of autophagosomes—temporary, large, and double-bilayered organelles in which materials are encapsulated for recycling. This chapter discusses what we know about lipid homeostasis and trafficking during autophagy and autophagosome formation and comments on future directions of the field.

Keywords: lipids, lipid homeostasis, lipid trafficking, autophagy, autophagosomes

1. Introduction

Careful control of cellular lipid pathways plays an important role in a cell's ability to maintain stable internal conditions in the face of an ever-changing extracellular environment. This is particularly true as it relates to cellular self-eating or autophagy, a process brought about by proteins collectively known as the Atg (in yeast) or ATG (in mammals, **A**u**T**opha**G**y) proteins [1]. Macroautophagy (herein referred to as autophagy) is the catabolic process by which unneeded or damaged cellular components are sequestered as cargo into unique double-membrane vesicles called autophagosomes [1]. Once made, autophagosomes deliver their contents for breakdown by docking and fusing with the cell's degradative organelle, the lysosome in animal cells or the vacuole in plant and yeast cells. Following the breakdown of these materials, the components can undergo efflux to enable recycling and reuse by cells. Autophagy can be activated in many ways, including by starvation and cellular damage. When induced by starvation, autophagy allows for the recycling of nutrients to sustain metabolism in the absence of extracellular nutrients. Under other conditions, such as when specific cellular components are damaged, autophagy can take more specific forms, targeting the damaged components for sequestration into autophagosomes and delivery to lysosomes/vacuoles for destruction and recycling of the generated materials. The damaged components sequestered by more targeted forms of autophagy include mitochondria (mitophagy), ribosomes (ribophagy), and peroxisomes (pexophagy) [2–4].

Dysregulation of autophagy has been linked to a variety of human disease states, including cancer, neurodegenerative disease, and heart disease [5]. Mutations in ATG genes can result in autosomal recessive human genetic conditions including diseases like Niemann-Pick Type C1, a progressive lipid storage disorder associated with impaired autophagosome maturation and characterized by neurodegeneration [6], Gaucher disease, a disorder related to the inability to breakdown specific cargo once autophagosomes fuse with the lysosome and characterized by hematologic symptoms [7], and Pompe disease, commonly coupled with glycogen accumulation in autophagic compartments and lysosomes, often resulting in myopathies [8, 9]. These relationships between autophagy and human disease have generated much interest in attaining a better understanding of autophagic processes, especially its hallmark—autophagosome formation [1, 5].

Autophagosomes differ from smaller, more traditional types of transport vesicles in that they form *de novo* (anew) rather than budding off of a pre-existing donor organelle, range from 600 to 900 nm rather than 60–100 nm in diameter, and are delimited by two bilayer membranes rather than by a single bilayer [1]. Autophagosome formation takes place at distinct cellular locations called pre-autophagosomal structures (PAS). Anew, membrane materials from different intracellular lipid sources are brought to the PAS to ultimately nucleate, form, and complete autophagosome biogenesis—highlighting the important role of lipid homeostasis in autophagy. Much effort has been dedicated to understanding the lipid trafficking events leading to autophagosome formation, the unique hallmark of autophagy. In this review, we discuss the progress that has been made in understanding this relationship between autophagosome formation and lipid homeostasis.

We begin this discussion by surveying the diversity of lipids present inside cells, including evidence on the role of each lipid type in autophagy. We discuss how membrane composition helps establish organelle identity, in order to place autophagosome formation within a larger context of lipid homeostasis. After considering how these lipids relate to autophagy, we discuss the membrane trafficking events leading to autophagosome formation. We conclude by commenting on the questions that remain unanswered at the intersection of autophagy and lipid trafficking.

2. Structure/function diversity of lipids and membranes in eukaryotes

Lipids are the only biologically relevant macromolecules that cannot be categorized as a polymer—their diversity is not dependent on monomer sequence like that of complex carbohydrates, proteins, or nucleic acids. The diversity of lipids is instead dependent on their chemical structure and the unique properties that are attained by combining different lipid molecules in the context of biological membrane bilayers [10–12]. The structural diversity of lipids in biological membranes can be divided into three different groups: glycerophospholipids (also often referred to as phospholipids), sphingolipids, and sterols (**Figure 1**). These three types of amphipathic lipids, when combined in different ratios and leaflet asymmetries, give rise to a variety of biological membrane bilayer properties such as fluidity and curvature. Lipids in biological membranes can be covalently modified through the attachment of carbohydrate or phosphate moieties through glycosylation or phosphorylation, respectively. These modified lipids also contribute to the functional diversity of biological membranes. The properties of a membrane bilayer are also influenced by the proteins embedded within it. Some transmembrane proteins can be post-translationally modified to contain chemical groups like carbohydrates

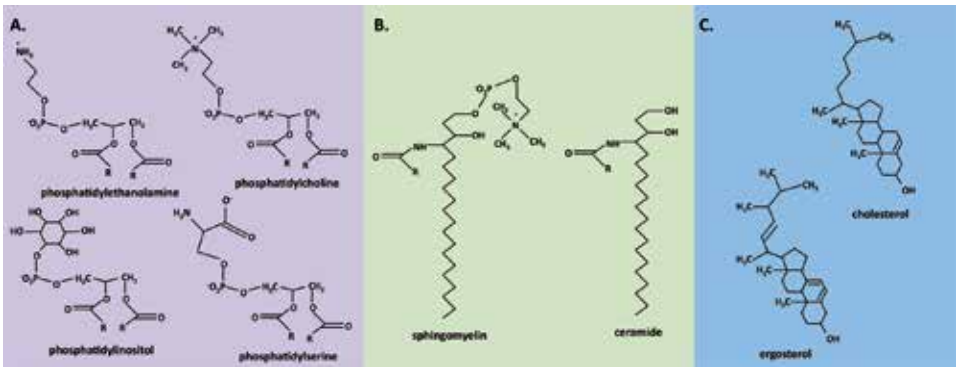

Figure 1.
Chemical structures of selected cell membrane lipids. Examples of some of the different types of lipids that can be found in cell membranes, namely (A) phospholipids, (B) sphingolipids, and (C) sterols.

(glycoproteins) and phosphate groups (phosphoproteins) that also inform membrane function. Some membrane and peripheral membrane proteins can be modified by covalent attachment to lipid groups as well.

2.1 Glycerophospholipids

Glycerophospholipids or phospholipids are the major component of biological membranes and their name evokes the building blocks used for their synthesis, including a three-carbon glycerol backbone (**Figure 1A**). This glycerol molecule is modified so that two of its hydroxyl groups (the ones attached to carbons 1 and 2) are esterified to covalently attach long-chain fatty acids to its backbone [10–12]. The fatty acid esterified to carbon 1 is often saturated (no carbon to carbon double-bonds) and the fatty acid esterified to carbon 2 is often unsaturated (with carbon to carbon double bonds present) [10–12]. Carbon to carbon double bonds in fatty acids have bends associated with them, which modify interactions with adjacent molecules and contribute to the fluidity of the bilayer [10–12]. The hydroxyl group on the third carbon of the glycerol backbone is covalently attached or esterified to phosphoric acid, adding a highly polar head group to the phospholipid and contributing to its amphipathic nature [10–12].

Glycerophospholipid diversity is determined by the specific fatty acids and potentially alcohol-modified phosphoric acid groups attached to the glycerol backbone. A cell can generate a large variety of more than 100 glycerophospholipids, each with a different combination of fatty acids on carbons 1 and 2 and a head group on carbon 3 [10–12]. Glycerophospholipids are named depending on the head group attached to carbon 3. Common glycerophospholipids in cells include: phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI) [10–12]. PE is the simplest phospholipid, with just a phosphoric acid moiety as a head group, while the other common types have alcohol-modified phosphoric acid head groups [10–12]. PI in particular is the kind of lipid that plays a major role in determination of organelle identity, as described later in this review [10–12].

Certain phospholipids and lipid-modifying enzymes are key for autophagy. For example, the PI3Kinase complex (Class III and potentially Class II) and its substrate PI are essential for autophagosome formation [13, 14]. Moreover, soluble autophagy-related proteins known to be required for the induction of autophagosome formation like Atg1 are known to be recruited to membrane enriched with PI and PI3P [13, 14].

Another lipid component essential for autophagosome formation is PE [13, 14]. PE lipids present in the autophagosome are important for the ability of this structure to associate with Atg8, another essential autophagy protein. This is because successful recruitment of Atg8 to autophagosomes involves Atg8 lipidation to the head group of PE, ultimately yielding a mature autophagosome that is decorated by lipidated Atg8 [13, 14]. In fact, PE is the lipid that contributes the most to autophagosome expansion [13, 14].

2.2 Sphingolipids

The backbone of sphingolipids is not glycerol but sphingosine, an amino alcohol synthesized from palmitoyl-CoA and serine [10–12]. The covalent attachment of the sphingosine backbone to a fatty acid through an amide linkage yields an amphipathic sphingolipid (**Figure 1B**) [10–12]. Common examples of this type of lipid in cells include ceramides (the simplest form of sphingolipid), phosphosphingolipids (also known as sphingomyelins, the only phospholipids without a glycerol backbone), and glycosphingolipids [10–12]. The differences and diversity between these sphingolipids is dependent on the type of fatty acid and additional head groups attached to the sphingosine backbone. Sphingolipids were discovered and can be predominantly found in nervous tissue. This type of lipid can also be found in lower eukaryotes such as budding yeast [10–12].

Sphingolipids such as sphingosine-1-phosphate (S1P) and ceramides have been found to have an effect on autophagy [13, 14]. S1P, as part of its well-known role in fostering proliferation and cell survival, can induce autophagy in a way that engages elements usually associated with apoptosis [13, 14]. Ceramides, in their well-known role in promoting cell cycle arrest, promote cell death while engaging elements associated with autophagy [13, 14].

2.3 Sterols

Sterols are four-ringed steroid molecules that contain a hydroxyl functional group at position 3 and as well as a variety of potential side chains (**Figure 1C**) [10–12]. These lipids can be present in plant, animal, and microbial cells such as budding yeast. The predominant form of sterol lipid in animal cells is cholesterol. In other organisms, the lipid forms most similar to cholesterol in function are called by different names (**Figure 1C**). For example, the functional yeast equivalent of cholesterol is referred to as ergosterol (**Figure 1C**).

Sterols in eukaryotes have been shown to influence specific forms of autophagy including chaperone-mediated autophagy (CMA), pexophagy (autophagy of peroxisomes) and lipophagy (autophagy of lipid droplets) [13, 14]. These types of specific autophagy are beyond the scope of this review and are therefore not discussed.

2.4 Lipid contributions to membrane structure/function

2.4.1 Membrane curvature

The concept of membrane curvature refers to the lipid composition asymmetry between the two leaflets of a membrane bilayer [11, 15, 16]. The membrane curvature of an area can be changed, not only by altering its lipid composition, but also through the function of specialized proteins that can bind and remodel membranes. Early autophagosomes, often referred to as phagophores, have a high degree of curvature and are enriched for PI and PI3P [13, 14]. This combination of curvature

and lipid composition is thought to help recruit a collective of autophagy-related proteins such as Atg1 and Atg3 to the site of autophagosome formation [13, 14].

2.4.2 Membrane fluidity

Membrane fluidity refers to the ability of a membrane to sustain diffusion-driven movement of molecules within it [11, 17]. In membranes, diffusion takes place laterally [11, 15, 17]. The presence of unsaturated and sterol lipid structures enhances membrane fluidity, ultimately allowing for more lateral diffusion of molecules and rendering it easier to bend and deform [11, 17].

This relationship between membrane fluidity and bending is highly relevant to autophagosome membranes [13, 14]. Early autophagosomal structures are characterized by high levels of curvature, suggesting that fluidity is key for autophagosome formation [13, 14]. In fact, autophagosomes are enriched for unsaturated lipids, and abrogation of enzyme functions that mediate desaturation can have inhibitory effects on autophagy [13, 14].

2.4.3 Lipid microdomains

Membranes are not even in composition throughout. Instead, they can have areas of differential lipid composition such as lipid rafts. Lipid rafts are generally more tightly packed (less fluid) than neighboring membrane material and their edges are usually rich in sphingolipids and cholesterol [11, 15, 17, 18]. Lipid rafts, because of their differential lipid composition, can act as scaffolds for non-clathrin-mediated internalization dynamics [11, 15, 17, 18].

While no specific lipid microdomains have been identified as characteristic of autophagosome membranes, it is known that lipid rafts associated with the endoplasmic reticulum and mitochondria can contribute to autophagosome formation from these lipid sources [13, 14].

3. Organelle lipid identity

Each membrane-bound organelle in the cell is characterized by a membrane bilayer with a particular lipid composition, leading to unique physical properties and the ability to recruit a specific set of interacting partners (**Table 1**). For this reason, organelle identity is not only defined by the collection of lipids displayed by the membrane of a particular organelle, but also by the set of proteins and molecules that are able to associate and interact with it. It is also important to recognize that these membranes can be remodeled and change their properties over time as cells strive to respond to stimuli.

Organelle Membrane	Lipid Content
Plasma membrane	ceramide, sphingosine, S_1P, diglyceride (also known as diacylglycerol), PI_4P, $PI_{(4,5)}P_2$, $PI_{(3,4)}P_2$,$PI_{(3,4,5)}P_3$
Endoplasmic Reticulum	PC, PE, PI, PS, PA, ceramide, galactosylceramide, cholesterol and ergosterol.
Mitochondria	PE, PG, cardiolipin, PA
Golgi	PE, PS, PC, PI_4P, sphingolipid
Late endosome	$PI_{(3,5)}P_2$, PI_3P, PS
Lipid Droplets	triacylglycerol esters and steryl esters, PE

Table 1.
Lipid content of different organelle membranes.

The two main molecular determinants of organelle membrane identity in cells are lipid content, specifically PIs, and association with activated small GTPases (such as the Rab family of proteins) [19, 20]. These membrane elements can easily be remodeled and changed, in contrast to transmembrane proteins and other integral membrane elements, allowing for the dynamic regulation and sculpting of membranes. Small GTPases exhibit diversity that correlates with specific subcellular locations, such that Rab 1 (ER and Golgi), Rab 2 (cis Golgi), Rab 4/11 (Recycling endosomes), and Rab 5 (Early endosomes, plasma membrane, clathrin coated vesicles) each localize to the membranes of different organelles [19, 20].

Small GTPases like Rabs exist in two forms: a GTP-bound active and membrane associated form, and a GDP-bound inactive cytosolic form. While Rabs can be lipidated (prenylated) at their C-terminus, facilitating their anchoring to the membrane, what is thought to determine Rab-membrane interaction specificity are the proteins that facilitate their GDP/GTP exchange [19, 20].

Organelle identity is also determined by specific forms of PI, with phosphate groups covalently linked to positions 3, 4, or 5 of their inositol ring [11, 12, 15, 19–22]. The plasma membrane contains predominantly PI4P and PI(4,5)P2, with the latter synthesized from the former [19–22]. These two can be phosphorylated by PI3-kinases (PI3K) to generate signaling lipids [19–22]. PI(4,5)P2 can also be cleaved to generate diacylglycerol (DAG; which can activate autophagic response) and I(1,4,5)P3 second messengers [19–22]. PI4P can also be found in the Golgi [19, 20]. Early endosomes are characterized by the presence of PI(3)P through the function of PI3-OH kinases like Vps34, which require small GTPases like Rab5 to function at the proper location [13, 14, 19–22]. This requirement for a Rab GTPase to produce the corresponding PI species characteristic of the compartment highlights the close functional relationship between the two key determinants of organelle identity [19–22]. The presence of PI(3,5)P (synthesized from PI3P) is characteristic of late endosomes [19–22].

The lipid composition of organelles is influenced by exchange of materials between them. There are different ways in which such exchanges can take place, including vesicle-mediated membrane trafficking between organelles, lipid droplet function, and direct exchange of lipid species via membrane contact sites between organelles such as the ER and Golgi. While the ER is the central site for intracellular lipid synthesis, lipid trafficking is essential for lipids to move from the ER to different organelles in order to maintain homeostatic membrane composition required for morphology, signaling and cellular processes. Moreover, it is common for organelles like the Golgi and endosome to change their lipid and membrane compositions as they mature.

Much of what we know about the lipid content of intracellular organelles or compartments is due to lipidomics studies that have served as a tool to identify lipid species in these membranes [23–27]. Biochemical fractionation and/or extraction techniques are used to isolate organellar membranes of interest and subject them to mass spectrometry for lipid identification and lipidome determination, similar to proteomics studies that have been used to catalog the protein content of many of these organelles [27]. Different efficiencies and membrane targets can be obtained depending on how the samples are prepared and treated during extraction leading up to ionization and mass spectrometry analysis [27]. Different types of data processing allow for a range of lipidomics applications, from species identification and quantification to pathway and network analysis [27]. These techniques have been thoroughly reviewed elsewhere and outside of the scope of this review article [27].

While proteomic studies have shed light on the proteins that reside in autophagosomes [28–31], an autophagosome lipidome has yet to be experimentally determined. Through microscopy and biochemical methods, we know that the lipid identity of autophagosomes is defined largely by the presence of PE, PI and PI3P.

In the following section of this review, we discuss in detail the molecular processes that bring about lipid homeostasis and trafficking during autophagy, particularly in autophagosome formation.

4. Understanding autophagosomes: a non-traditional membrane vesicle

Current efforts to better understand autophagy are focused on the molecular details leading to the formation of autophagosomes, a double-membraned vesicle unlike any other in size and structure, that serves as the hallmark of autophagy. These approaches have mainly focused on understanding the intracellular trafficking of autophagy-related transmembrane proteins that are thought to help deliver lipids to the growing autophagosome via vesicle transport. Insights have also been obtained from assessing the influence of lipid-dependent enzymes as well as lipid-synthesis and transport pathways on autophagy. For example, mutation of PI3K enzymes severely impairs autophagy [32, 33], pointing at the importance PI and some of its phosphorylated forms in autophagic processes. In these ways, we have identified that PE and PI lipids play an important role in autophagy (**Table 2**) [14, 34–41]. We discuss these findings below.

4.1 Membrane dynamics during autophagy

The process of autophagy is often thought about to happen in different stages—initiation or nucleation of phagophores (cup-shaped, double-membraned autophagosome precursors), expansion of phagophores, completion or closure of autophagosomes, fusion of autophagosomes with the lysosome/vacuole, and the efflux of materials from the lysosome/vacuole for reuse (**Figure 2**). Because our review aims to focus on the lipid homeostasis and trafficking during autophagy, we focus our discussion below on the first four stages of autophagy (initiation through fusion).

4.1.1 Initiation

During the initial stages of autophagy, essential Atg proteins are recruited to PAS structures to nucleate the gathering of membrane *de novo* and to generate

Donor Membrane	Lipid Contributions	Autophagic Process/Step Benefited	References
Endoplasmic Reticulum	PI_3P	Autophagosome formation	34
Mitochondria	PE	Autophagosome formation	35
Plasma Membrane	PI_3P	Autophagosome and ATG16 vesicle formation	36
Golgi	PI_4P, PE, PS	ATG9 vesicles formation, autophagosome fusion with vacuole/lysosome	37-39
Late Endosome	PI_3P, $PI_{(3,5)}P_2$, PS	Autophagosome formation, autophagosome fusion with vacuole/lysosome	39, 40
ER-Golgi Intermediate compartments	PE	LC3/Atg8 lipidation	41
ER-Mitochondria contact sites	PE	Autophagosome formation	42
ER-Plasma membrane contact site	PI_3P	Autophagosome formation	43

Table 2.
Organellar lipid contributions to autophagic processes and autophagosome formation.

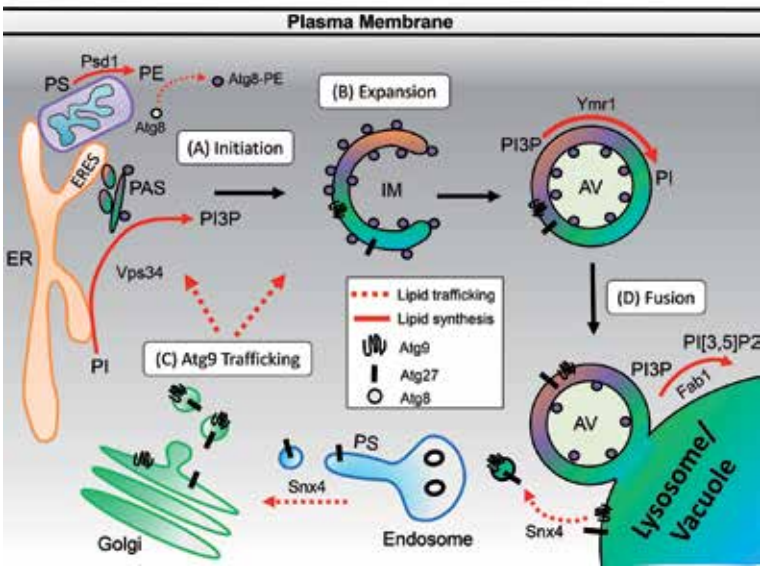

Figure 2.
Lipid biosynthesis and trafficking during autophagosome biogenesis and fusion. The relative lipid contributions via lipid trafficking or lipid biosynthesis from each organelle are shown. (A) upon autophagy induction, membrane is sequestered from pools of lipid donors such as the ER, mitochondria, Golgi, endosome and vacuole to form the pre-autophagosomal structure (PAS) or isolation membrane (IM). (B) PI in the PAS is converted to PI3P via Vps34 and Atg8-PE is enriched, allowing for IM expansion and closure. (C) Additional lipid membrane from Atg9-Atg27 vesicle trafficking allows for further expansion of the IM. (D) Autophagosome (AV) maturation is completed once PI3P is converted into PI by myotubularin phosphatases such as Ymr1. The mature AV can fuse to the vacuole which has converted PI3P into PI(3,5)P2 via Fab1 kinase. PI(3,5)P2 permits fusion of AV and endosomes. Vacuole lipid homeostasis is further aided by Snx4 trafficking of PS and Atg27 from the endosome and vacuole.

autophagosome precursors called phagophores. In mammalian cells, phagophores can be formed at PAS sites proximal to ER, mitochondria, or plasma membrane [13, 14]. In yeast however, PAS structures are perivacuolar in nature, leading phagophores to originate at locations proximal to the vacuole with membrane contributions with other cellular locations like discussed below. While mammalian cells can display many PAS structures at steady state, yeast often display one of these structures at any given time.

Initially, activated or triggered by upstream inhibition of mTOR, core autophagy proteins collect at the PAS structure. These core factors include the ULK1 complex, ATG13, FIP200, ATG101, (yeast Atg1, Atg13, Atg17, Atg29 and Atg31) [13, 14]. The mobilization of these factors ultimately leads to the PAS recruitment and activation of the PI3K/Vps34 kinase complex which allows for the generation of PI3P at this site (from PI) (**Figure 2A**) [13, 14, 30, 31]. This enzyme activity drives forward phagophore initiation/nucleation, allowing additional recruitment of other proteins like WIPI proteins (Atg18 in yeast). WIPI proteins are key PI3P effectors. PI3K regulator proteins like ATG14 (Atg14 in yeast) are also recruited to the PAS, allowing for the modulation of initiation/nucleation of phagophore formation [13, 14]. ATG14 is also able to mediate homotypic fusion of single-membrane vesicles at the PAS, allowing more traditional membrane vesicles delivered to the PAS to fuse and contribute to the nucleation and growth of the phagophore [13, 14]. In this way, the phagophore has been found to accept lipid inputs from the ER, ER exit sites (ERES), the Golgi, the plasma membrane and recycling endosomes for its growth and expansion (**Figure 2A**) [13, 14].

Generation of PI3P at PAS sites functions as a scaffold for ATG/Atg proteins to associate with the PAS, helping in the expansion, elongation and curvature generation needed for autophagosome formation to proceed successfully [13, 14].

4.1.2 Expansion

The lipidation of Atg8 (LC3) onto PE lipid molecules in the growing phagophore membrane is key for autophagosome formation. Other Atg proteins like ATG16L1 (yeast Atg16) as well as ATG12-ATG5 are required for Atg8 lipidation with PE. Interestingly, some of these required Atg factors like ATG16L1 partly reside in the recycling endosome and have been found to localize to the PAS in a PX-BAR/SNX protein-dependent manner (for example, SNX18) [13, 14]. While not considered core Atg proteins, PX-BAR/SNX proteins are membrane remodeling proteins that have also been found to be required for autophagosome formation.

While the exact lipid composition of the phagophore/autophagosome is still under debate, because of the findings described above, PI3P is thought to be one of the most abundant lipid species present in these structures. In fact, recent findings identify the ATG protein ATG2A as a lipid shuttle factor that facilitates PI3P-dependent autophagosome growth [42–44]. Other phosphoinositides such as PI4P, PI(4,5)P2 and PI(3,5)P2 have also been suggested to play a role in the expansion of the phagophore [13, 14]. Apart from these, as mentioned above, sphingosine-1-phosphate (S1P) and ceramide, also contribute to autophagy. And while, Atg8/LC3 lipidation resulting in LC3-PE is important for the expansion and closure of the autophagosome, the intracellular membranes that are the source of this PE still remain to be identified. PE is produced from phosphatidylserine (PS) in mitochondria, which might be one of the sources of PE (**Figure 2A**). PE might also be shuttled from the ER, plasma membrane and recycling endosomes. It has been recently shown by Ma et al. [39], that PS trafficking by sorting nexin Snx4 in yeast is required for maintaining the correct lipid composition of the vacuole for autophagosome fusion in yeast (**Figure 2C**). Thus, maintenance of organelle lipid identity through proper trafficking and lipid homeostasis are crucial for autophagy as well.

4.1.3 ATG9 trafficking

ATG9 (Atg9 in yeast) is a six-transmembrane protein that is required for autophagosome formation [45–52]. ATG9 trafficking is one of the most studied topics in the autophagy field, highlighting the important role it plays in autophagy—the autophagic phenotypes of null mutants are very penetrant. While this is the case, we still lack detailed information about the function of this protein during autophagy. Yeast studies have shown that Atg9 is localized to PAS as well as cytoplasmic vesicles of 30–60 nm diameter that bud-off of the late Golgi. It is believed that Atg9 shuttles between the PAS and its cytoplasmic vesicle pool upon autophagy induction (**Figure 2C**). At the PAS, Atg9 associates with Atg1, Atg2 as well as Atg18. Since Atg9 is a transmembrane protein, its trafficking directly affects the funneling or channeling of membrane to the PAS and the growing autophagosome. The Atg9 vesicles are thought to originate at the Golgi and contain fusion factors such as subunits of the TRAPIII (Trs85) complex that are responsible for fusion with the growing autophagosome [45, 46, 52]. Once Atg9 reaches the lysosome/vacuole as autophagosomes fuse, some studies suggest that the protein can be recycled out of the vacuolar membrane for reuse in new rounds of autophagosome formation. These studies are synergistic with findings that Atg9 traffics through endosomal compartments, as this might be an intermediary step important for recycling.

This recycling model is supported by the observation that mutations in the retromer complex, when combined with mutations in tethering factor Trs85, can abrogate trafficking of Atg9 to the PAS. Similarly the combination mutations with GARP subunits that are responsible for tethering vesicles to the Golgi from the endosome, with Trs85 result in defective autophagy. Atg9 trafficking is influenced by other autophagy proteins such as Atg27 and Atg23 [53–56]. Atg23 is a peripheral membrane protein that facilitates the anterograde trafficking of Atg9 from the Golgi to the PAS. Atg27, another transmembrane protein that facilitates formation of Atg9 vesicles at the late Golgi, is itself observed to be present in early/late endosomes, PAS, Golgi and vacuolar membrane. Atg27 can be retrieved from the vacuole in a process that is Snx4-dependent. Furthermore, earlier studies identified a C terminal tyrosine (YSAV) motif in Atg27 that is important for the proper delivery of Atg27 to the vacuole as well as to maintain Atg9 pools at the endosome that can be mobilized to different compartments during autophagy (**Figure 2C**). Taken together, the complexity of Atg9 trafficking synergizes with the hypothesis that a collection of different membranes such as endosome and Golgi all contribute to the lipid identity of the autophagosome.

4.1.4 Autophagosomal fusion with the lysosome/vacuole

After the autophagosome is completed, the double membrane vesicle is ready for fusion with the lysosome/vacuole. The fusion of the autophagosome not only requires components of the fusion machinery as well as PI3P turnover, but it also requires the disassembly or retrieval of several ATG proteins from the autophagosome. In yeast, phosphoinositide phosphatases, including those from the myotubularin protein family, like Ymr1, along with Sjl2 and Sjl3 are important for removal of PI3P from completed autophagosomes, making autophagosomes fusion-competent (**Figure 2D**). In mammalian cells, PI3P phosphatase MYM-3 acts similarly to Ymr1 in promoting autophagosome maturation and fusion. Fusion of autophagosomes with the lysosome is mediated by RAB7-like protein Ypt7 along with the HOPS (Homotypic fusion and vacuole protein sorting) tethering complex and SNARE (Soluble Ethylmaleimide-Sensitive Factor Attachment Protein Receptor) proteins. Three Q–SNAREs: Vam3, Vti1 and Vam7 have been identified in yeast along with R-SNARE Ykt6 as key for this fusion step. Vamp7 is a sorting nexin family protein containing a PX domain that interacts with PI3P. Studies suggest that Vam7 interacts with Atg17-Atg29-Atg31 trimer complex via Atg17 interaction. In mammalian cells, SNAREs such as SYN12, SNAP29 and VAMP7/8 are responsible for autophagosome fusion with the lysosome. Interestingly, the PI3K VPS34 has also been linked with later stages of autophagy, including lysosomes returning to normal or regenerating once fusion with autophagosomes has taken place. Other phosphorylated lipid species like PI4P and PI(4,5)P2 can also facilitate lysosomal regeneration, allowing for new rounds of autophagy to occur. This might also trigger the formation of recycling vesicles packaging ATG protein cargo for recycling off the lysosomal membrane.

Another aspect that is key for late autophagy stages to proceed is intact lysosomal/vacuolar lipid homeostasis and lipid identity. For example, yeast vacuoles have unique lipid composition that is different from other membrane organelles within the cell. Vacuoles are enriched with lipids such as, myo-inositols, PI3P, and typically PI3,5P2, among other lipids such as ergosterol, diacylglycerol and some sphingolipids (**Figure 2D**). The specific lipid identity of vacuoles is important for the recruitment of fusion factors such as SNARES Ypt7 and HOPS that allow fusion of autophagosomes. Thus, the specific lipid identity of vacuole is important for its physiological function and its fusion with the late endosome and autophagosome.

5. Conclusion

While progress has been made in understanding the molecular underpinnings of autophagosome formation, our understanding has been primarily advanced by understanding the functions of proteins that lead and are required for this autophagic vesicle to form [28–31]. Because the extensive level of membrane remodeling that takes place during autophagy formation, much will be gained by investigating the process using methods that focus on the membrane and lipid biology of the process [23–27]. This new perspective has the potential of changing the way we have conventionally understood the remodeling of membranes for vesicle formation.

Table 2 describes the lipid contributions of different intracellular organelles or donor membranes to the progression of autophagy and/or autophagosome formation. The autophagy processes or steps benefiting from these contributions are also indicated.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

Atg/ATG proteins	autophagy-related proteins
PAS	pre-autophagosomal structure
PA	phosphatidic acid
PG	phosphatidylglycerol
PE	phosphatidylethanolamine
PC	phosphatidylcholine
PS	phosphatidylserine
PI	phosphatidylinositol
PI3P	phosphatidylinositol-3-phosphate
PI (3,5) P2	phosphatidylinositol-3, 5-biphosphate
PI4P	phosphatidylinositol-3-phosphate
PI(4,5)P2	phosphatidylinositol-4, 5-biphosphate
PI3K	phosphatidylinositol-3-kinase
S1P	sphingosine-1-phosphate

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References

[1] Yang Z, Klionsky DJ. Eaten alive: A history of macroautophagy. Nature Cell Biology. 2010;**12**(9):814

[2] Wang K, Klionsky DJ. Mitochondria removal by autophagy. Autophagy. 2011;**7**(3):297-300

[3] Cebollero E, Reggiori F, Kraft C. Reticulophagy and ribophagy: Regulated degradation of protein production factories. International Journal of Cell Biology. 2012;**2012**:182834

[4] Sakai Y, Oku M, van der Klei IJ, Kiel JA. Pexophagy: Autophagic degradation of peroxisomes. Biochimica et Biophysica Acta, Molecular Cell Research. 2006;**1763**(12):1767-1775

[5] Choi AM, Ryter SW, Levine B. Autophagy in human health and disease. The New England Journal of Medicine. 2013;**368**(7):651-662

[6] Sarkar S, Carroll B, Buganim Y, Maetzel D, Ng AH, Cassady JP, et al. Impaired autophagy in the lipid-storage disorder Niemann-pick type C1 disease. Cell Reports. 2013;**5**(5):1302-1315

[7] Ivanova MM, Changsila E, Iaonou C, Goker-Alpan O. Impaired autophagic and mitochondrial functions are partially restored by ERT in Gaucher and Fabry diseases. PLoS One. 2019;**14**(1):e0210617

[8] Raben N, Roberts A, Plotz PH. Role of autophagy in the pathogenesis of Pompe disease. Acta Myologica. 2007;**26**(1):45

[9] Lim JA, Sun B, Puertollano R, Raben N. Therapeutic benefit of autophagy modulation in Pompe disease. Molecular Therapy. 2018;**26**(7):1783-1796

[10] Harayama T, Riezman H. Understanding the diversity of membrane lipid composition. Nature Reviews. Molecular Cell Biology. 2018;**19**(5):281

[11] Voet D, Voet JG, Pratt CW. Fundamentals of Biochemistry: Life at the Molecular Level. 5th ed. New Jersey: Wiley; 2016

[12] Berg JM, Tymoczko JL, Stryer L. Biochemistry. 8th ed. New York: W H Freeman; 2015

[13] Knævelsrud H, Simonsen A. Lipids in autophagy: Constituents, signaling molecules and cargo with relevance to disease. Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids. 2012;**1821**(8):1133-1145

[14] Dall'Armi C, Devereaux KA, Di Paolo G. The role of lipids in the control of autophagy. Current Biology. 2013;**23**(1):R33-R45

[15] Ridgway N, McLeod R, editors. Biochemistry of Lipids, Lipoproteins and Membranes. 6th ed. Amsterdam: Elsevier; 2015

[16] McMahon HT, Boucrot E. Membrane curvature at a glance. Journal of Cell Science. 2015;**128**(6):1065-1070

[17] Beney L, Gervais P. Influence of the fluidity of the membrane on the response of microorganisms to environmental stresses. Applied Microbiology and Biotechnology. 2001;**57**(1-2):34-42

[18] Edidin M. Lipid microdomains in cell surface membranes. Current Opinion in Structural Biology. 1997;**7**(4):528-532

[19] Behnia R, Munro S. Organelle identity and the signposts for membrane traffic. Nature. 2005;**438**(7068):597

[20] Alberts B, Bray D, Hopkin K, Johnson AD, Lewis J, Raff M, et al.

Essential Cell Biology. 5th ed. New York: W. W. Norton; 2019

[21] van Meer G, Voelker DR, Feigenson GW. Membrane lipids: Where they are and how they behave. Nature Reviews. Molecular Cell Biology. 2008;**9**(2):112

[22] van Meer G, de Kroon AI. Lipid map of the mammalian cell. Journal of Cell Science. 2011;**124**(1):5-8

[23] Brügger B. Lipidomics: Analysis of the lipid composition of cells and subcellular organelles by electrospray ionization mass spectrometry. Annual Review of Biochemistry. 2014;**83**:79-98

[24] Klose C, Surma MA, Simons K. Organellar lipidomics—Background and perspectives. Current Opinion in Cell Biology. 2013;**25**(4):406-413

[25] Loizides-Mangold U. On the future of mass-spectrometry-based lipidomics. The FEBS Journal. 2013;**280**(12):2817-2829

[26] Aviram R, Manella G, Kopelman N, Neufeld-Cohen A, Zwighaft Z, Elimelech M, et al. Lipidomics analyses reveal temporal and spatial lipid organization and uncover daily oscillations in intracellular organelles. Molecular Cell. 2016;**62**(4):636-648

[27] Yang K, Han X. Lipidomics: Techniques, applications, and outcomes related to biomedical sciences. Trends in Biochemical Sciences. 2016;**41**(11):954-969

[28] Øverbye A, Brinchmann MF, Seglen PO. Proteomic analysis of membrane-associated proteins from rat liver autophagosomes. Autophagy. 2007;**3**(4):300-322

[29] Shui W, Sheu L, Liu J, Smart B, Petzold CJ, Hsieh TY, et al. Membrane proteomics of phagosomes suggests a connection to autophagy. Proceedings of the National Academy of Sciences. 2008;**105**(44):16952-16957

[30] Dengjel J, Høyer-Hansen M, Nielsen MO, Eisenberg T, Harder LM, Schandorff S, et al. Identification of autophagosome-associated proteins and regulators by quantitative proteomic analysis and genetic screens. Molecular and Cellular Proteomics. 2012;**11**(3):M111-M014035

[31] Zimmermann AC, Zarei M, Eiselein S, Dengjel J. Quantitative proteomics for the analysis of spatio-temporal protein dynamics during autophagy. Autophagy. 2010;**6**(8):1009-1016

[32] Diao J, Liu R, Rong Y, Zhao M, Zhang J, Lai Y, et al. ATG14 promotes membrane tethering and fusion of autophagosomes to endolysosomes. Nature. 2015;**520**(7548):563

[33] Ma M, Burd CG, Chi RJ. Distinct complexes of yeast Snx4 family SNX-BARs mediate retrograde trafficking of Snc1 and Atg27. Traffic. 2017;**18**(2):134-144

[34] Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, et al. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. The Journal of Cell Biology. 2008;**182**(4):685-701

[35] Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, et al. Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell. 2010;**141**(4):656-667

[36] Ravikumar B, Moreau K, Jahreiss L, Puri C, Rubinsztein DC. Plasma membrane contributes to the formation of pre-autophagosomal structures. Nature Cell Biology. 2010;**12**(8):747

[37] Mari M, Griffith J, Rieter E, Krishnappa L, Klionsky DJ, Reggiori F. An Atg9-containing compartment that functions in the early steps of autophagosome biogenesis. The Journal of Cell Biology. 2010;**190**(6):1005-1022

[38] Judith D, Jefferies HB, Boeing S, Frith D, Snijders AP, Tooze SA. ATG9A shapes the forming autophagosome through Arfaptin 2 and phosphatidylinositol 4-kinase IIIβ. The Journal of Cell Biology. 2019;**218**(5):1634-1652

[39] Ma M, Kumar S, Purushothaman L, Babst M, Ungermann C, Chi RJ, et al. Lipid trafficking by yeast Snx4 family SNX-BAR proteins promotes autophagy and vacuole membrane fusion. Molecular Biology of the Cell. 2018;**29**(18):2190-2200

[40] Ge L, Melville D, Zhang M, Schekman R. The ER–Golgi intermediate compartment is a key membrane source for the LC3 lipidation step of autophagosome biogenesis. eLife. 2013;**2**:e00947

[41] Nascimbeni AC, Giordano F, Dupont N, Grasso D, Vaccaro MI, Codogno P, et al. ER–plasma membrane contact sites contribute to autophagosome biogenesis by regulation of local PI3P synthesis. The EMBO Journal. 2017;**36**(14):2018-2033

[42] Osawa T, Noda NN. Atg2: A novel phospholipid transfer protein that mediates de novo autophagosome biogenesis. Protein Science. 2019;**28**(6):1005-1012

[43] Maeda S, Otomo C, Otomo T. The autophagic membrane tether ATG2A transfers lipids between membranes. eLife. 2019;**8**:e45777

[44] Valverde DP, Yu S, Boggavarapu V, Kumar N, Lees JA, Walz T, et al. ATG2 transports lipids to promote autophagosome biogenesis. The Journal of Cell Biology. 2019;**218**(6):1787-1798

[45] Yamamoto H, Kakuta S, Watanabe TM, Kitamura A, Sekito T, Kondo-Kakuta C, et al. Atg9 vesicles are an important membrane source during early steps of autophagosome formation. The Journal of Cell Biology. 2012;**198**(2):219-233

[46] Kakuta S, Yamamoto H, Negishi L, Kondo-Kakuta C, Hayashi N, Ohsumi Y. Atg9 vesicles recruit vesicle-tethering proteins Trs85 and Ypt1 to the autophagosome formation site. The Journal of Biological Chemistry. 2012;**287**(53):44261-44269

[47] Suzuki SW, Yamamoto H, Oikawa Y, Kondo-Kakuta C, Kimura Y, Hirano H, et al. Atg13 HORMA domain recruits Atg9 vesicles during autophagosome formation. Proceedings of the National Academy of Sciences of the United States of America. 2015;**112**(11):3350-3355

[48] Karanasios E, Walker SA, Okkenhaug H, Manifava M, Hummel E, Zimmermann H, et al. Autophagy initiation by ULK complex assembly on ER tubulovesicular regions marked by ATG9 vesicles. Nature Communications. 2016;**7**:12420

[49] Rao Y, Perna MG, Hofmann B, Beier V, Wollert T. The Atg1–kinase complex tethers Atg9-vesicles to initiate autophagy. Nature Communications. 2016;**7**:10338

[50] Reggiori F, Tooze SA. Autophagy regulation through Atg9 traffic. The Journal of Cell Biology. 2012;**198**(2):151-153

[51] Orsi A, Razi M, Dooley HC, Robinson D, Weston AE, Collinson LM, et al. Dynamic and transient interactions of Atg9 with autophagosomes, but not membrane integration, are required for autophagy. Molecular Biology of the Cell. 2012;**23**(10):1860-1873

[52] Shirahama-Noda K, Kira S, Yoshimori T, Noda T. TRAPPIII is responsible for vesicular transport from early endosomes to Golgi, facilitating Atg9 cycling in autophagy. Journal of Cell Science. 2013;**126**(21):4963-4973

[53] Yen WL, Legakis JE, Nair U, Klionsky DJ. Atg27 is required for autophagy-dependent cycling of Atg9. Molecular Biology of the Cell. 2007;**18**(2):581-593

[54] Yen WL, Klionsky DJ. Atg27 is a second transmembrane cycling protein. Autophagy. 2007;**3**(3):254-256

[55] Backues SK, Orban DP, Bernard A, Singh K, Cao Y, Klionsky DJ. Atg23 and Atg27 act at the early stages of Atg9 trafficking in *S. cerevisiae*. Traffic. 2015;**16**(2):172-190

[56] Segarra VA, Boettner DR, Lemmon SK. Atg27 tyrosine sorting motif is important for its trafficking and Atg9 localization. Traffic. 2015;**16**(4):365-378

Chapter 7

Cell-Based Assays in Cancer Research

Biba Vikas and Sukumaran Anil

Abstract

Cell growth is referred to as cell proliferation, that is, the increase in cell numbers during repeated cell division. Cell growth can be defined as the enlargement of cell volume, which might take place in the absence of cell division. Growth and reproduction are features of cells in all living organisms. All cells reproduce by dividing into two, with each parental cell giving rise to two daughter cells each time they divide. Various genes are involved in the control of cell division and growth. Reproduction in unicellular organisms are referred to cell division and in multicellular organisms it is tissue growth and maintenance. Survival of the eukaryotes depends upon interactions between various cell types, that helps in the balanced distribution. This is achieved through the highly regulated process of cell proliferation. Knowledge in cell cycle is necessary to determine the best time to collect cells, to harvest cell products, or to move cells to a new growth environment. Cancer cells do not die at the natural point in a cell's life cycle. Cancer cells occur as the results of cellular changes caused by the uncontrolled growth and division of cells. The chapter focuses on cancer cell maintenance, apoptosis, and its detection assays.

Keywords: cancer cell maintenance, apoptosis, cytotoxicity, cell-based assays

1. Introduction

Cell division and the increase in cell quantity is called cell growth. Cell growth happens in favorable nutrient conditions. It is the process by which cells accumulate mass and increase in physical size. The growth of tissues that are not self-renewing occurs by a combination of increase in number and increase in size of the component cells. Cells will progress unimpeded through the cell cycle and divide; one cell will become two, two will become four, four will become eight, and so on. Cellular growth is ensured by the alternation of DNA duplication and cell division cycles [1]. When the cell reaches maximum size, the important point is that the surface area to the volume ratio becomes smaller as the cell gets larger. When the cell grows beyond a certain limit, inadequate material will be able to cross the membrane sufficient to accommodate the increased cellular volume. Increase of size and change in shape of a developing organism depend on the increase in the number and size of cells. The increase in cell number is due to cellular reproductive mechanism called mitosis. Cells are limited to their programmed size because the cell membrane must transport oxygen and food into the cell, as well as transport waste like CO_2 and H_2O out of the cell. As the cell grows, the inside grows faster than the outside. Cellular growth is ensured by the alternation of DNA duplication and cell division cycles. As the cell grows, the inside grows faster than the outside. Cellular growth is ensured

by the alternation of DNA duplication and cell division cycles; through transcription the coupling of cell divisions is taking place in metabolic pathways [2].

Cancer cell growth: cancer is a condition where cells grow uncontrollably in a specific part of the body. Cancerous cells can invade and destroy surrounding healthy tissue and organs. Cancer cells divide relentlessly, forming solid tumors or flooding the blood with abnormal cells. Cell division is a normal process for growth and repair. A parent cell divides to form two daughter cells and is used to build new tissue, or it is used to replace cells that have died because of aging or damage. Healthy cells stop dividing when there is no longer a need for more daughter cells, but cancer cells continue the divisions. They are capable of spreading from one part of the body to another in a process known as metastasis [3]. Cell proliferation and cell death are such diametrically opposed cellular fates that are linked and interdependent processes [4, 5].

Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a suitable artificial environment. In vitro assays are performed to check the proliferation that reflects cellular responses to various stimuli. These techniques help to observe cell division and quantity of cells. Cell culture technology shows a good progress in biology and is heavily dependent on cell culture technology. Chemotherapeutic agent phenoxodiol, a synthetic analog of daidzein, a well-known isoflavone from soybean (*Glycine max*) was developed as a therapeutic agent against cervical, ovarian, prostate, renal, and vaginal cancers. They induce apoptosis through the inhibition of antiapoptotic proteins [6]. Apoptotic shrinkage, disassembly into apoptotic bodies, and engulfment of individual cells characteristically occur without associated inflammation. This could then be the consequence of releasing intracellular contents into tissues. This could be the consequence of releasing intracellular contents into tissues; the mitochondria remain unchanged morphologically [7].

1.1 The intrinsic pathway for programmed cell death

The signaling pathway for programmed cell death involves non-receptor-mediated intracellular signals inducing activities in the mitochondria that initiate apoptosis. Stimuli for the intrinsic pathway are caused by viral infections or damage to the cell by toxins, free radicals, or radiation. Damage to the cellular DNA can also induce the activation of the intrinsic pathway for programmed cell death [8]. Proapoptotic proteins activate caspases to mediate the destruction of the cell through different pathways. These proteins translocate to the nucleus of cells, thereby inducing DNA fragmentation which is a hallmark of apoptosis. The members of the Bcl-2 family of proteins and the tumor suppressor protein p53 regulate proapoptotic event in the mitochondria (**Figure 1a**). The Bcl-2 family members of proteins may be pro- or antiapoptotic. Bcl-2, Bcl-x, Bcl-xL, Bcl-XS, Bcl-w, and BAG are the antiapoptotic proteins. These proteins are currently under investigation as potential targets for anticancer therapy. Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk are proapoptotic proteins. The upregulation of these proteins or their increased activation may offer an approach for cancer therapy [9]. Cellular pathways that modulate the activities of the p53 protein are also currently being evaluated as targets for potential anticancer therapies [10].

1.2 The extrinsic pathway for programmed cell death

The extrinsic signaling pathway leads to apoptosis that is through transmembrane death receptors, members of the tumor necrosis factor (TNF) receptor gene super family. Members of this receptor family bind to extrinsic ligands and

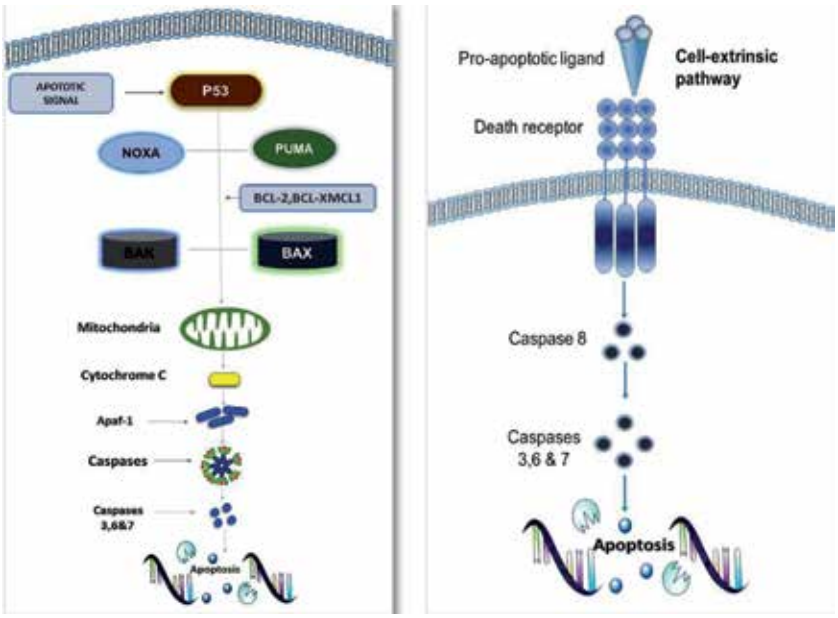

Figure 1.
(a) The intrinsic pathway for programmed cell death. (b) The extrinsic pathway for programmed cell death.

transduce intracellular signals that result in the destruction of the cell [11]. The most well-characterized ligands of these receptors are FasL, TNF-alpha, Apo3L, and Apo2L, and its corresponding receptors are FasR, TNFR1, DR3, and DR4/DR5, respectively [8, 9, 12]. Molecules that stimulate the activity of these proapoptotic proteins or activate these receptors are currently under the evaluation for their therapeutic potential in the treatment of cancer, including hematologic malignancies. The signal transduction of extrinsic pathway involves various caspases which are proteases with specific cellular targets. Once activated, the caspases affect several cellular functions as part of a process that results in the death of the cells [8]. This visible transformation of apoptosis is accompanied by biochemical changes. Those at the cell surface include the externalization of phosphatidyl serine and other alterations that promote recognition by phagocytes. Intracellular changes include the degradation of the chromosomes of the chromosomal DNA into high molecular weight and oligonucleosomal fragments and cleavage of a specific subset of cellular polypeptide [13, 14]. These cleavages are accompanied by a family of intracellular proteases called caspases (**Figure 1b**).

2. 2D and 3D cell culture systems

Cell culture is used in vitro in cell biology, tissue morphology, and mechanisms of diseases, drug action, protein production, and the development of tissue engineering. The stage of the culture after the cells can be isolated from the tissue and proliferates under favorable conditions until they reach confluency. In this stage, the cells have to be passaged or subcultured by transferring them to a new vessel with fresh growth medium to provide more room for continued growth. Various researches in cancer biology are based on experiments using two-dimensional cell culture by growing cells in flat dishes, made of plastic. The dish culture system is mainly used for developing adherent two-dimensional cell monolayers. 3D cell culture applications are usually beneficial in tissue engineering and regenerative

medicine. 3D cell culture is an artificially created environment in which biological cells are allowed to grow and interact with their surroundings in all three dimensions. These three-dimensional cultures are grown in bioreactors, small capsules in which the cells can grow into spheroids, otherwise 3D cell colonies. Approximately 300 spheroids are usually cultured per bioreactor 3D cell culture which allows cells in vitro to grow in all directions, similar to how they would in vivo [15]. An increasing shift in research is occurring, where 3D cell culture systems are replacing 2D cell culture systems and translating 2D in vitro research to 3D before or as an alternative to testing using in vivo animal models [16, 17].

2.1 Preparation of culture media

Culture media provide artificial environment to grow the cancer cells in vitro. Culture media can be prepared by mixing DMEM powder (glucose, L-glutamine, pyridoxine, HCl, without pyruvate) in autoclaved triple distilled water. To this 1.95 g of HEPES buffer and 3.75 g sodium bicarbonate can be added. Antibiotics such as penicillin (500 µl) and streptomycin (500 µl) and fungicide-amphotericin-B (750 µl) can also be added. The volume can then be made up to 1000 ml, and the pH will adjust to 7.2–7.4. The medium will then filter under negative pressure using 0.22 µm cellulose filter. Sterility of the medium can be tested before use. Ten percent FCS can be mixed with the medium prior to culture.

2.2 Maintenance of adherent cancer cell lines

Adherent cell lines will grow in vitro until they form a monolayer over surface area available or medium depleted of nutrients. Adherent cells human oral cancer cells (KB), lung adenocarcinoma (A-549), and breast cancer (MCF-7) can be cultured in tissue culture flasks. The cells were disaggregated by trypsinization and subcultured when the monolayer reaches about 70% confluency. The cells will be cryopreserved at −80°C. With an inverted microscope, the degree of confluency of the cell monolayer can be assessed, and the absence of bacterial and fungal contaminants can be confirmed. Spent medium can be removed. Cells can be washed with PBS-EDTA for removing the traces of serum. Trypsin/EDTA (500 µl) will be applied onto the cell monolayer, and the flask is swirled to cover the monolayer with trypsin. The flask will be incubated at 37°C for 2–3 min. The flask can be examined under the inverted microscope to ensure uniform detachment of the cells. 1–2 ml of medium can be added to the flask as fast as possible to lessen the trypsin-induced stress, and the contents of the flask can be transferred to a centrifuge tube. Cells should be centrifuged at 1500 rpm, for 10 min. The supernatant will be discarded, and the cells were resuspended in minimum volume of medium. Cells can be counted using a hemocytometer, and the required numbers of cells can be subcultured in a new flask containing fresh DMEM with 10% FCS. This process can be repeated as demanded by the growth characteristics of the cell line.

2.3 Maintenance of cancer suspension cell lines

In general terms cultures derived from blood (e.g., lymphocytes grow in suspension) cells may be seen as single cells or clumps. For these types of cell lines, subculturing is done by dilution in small volume of media before counting. The culture can be viewed using an inverted phase contrast microscope; cells growing in exponential phase should be bright, round, and refractive. Cell suspension can be mixed well and dispersed uniformly by repeated pipetting in order to make single-cell suspension. The cells can be counted, and 1×10^6 cells can be seeded to a fresh

bottle containing 10 ml of DMEM medium with 10% FBS antibiotics and incubated at 37°C and subcultured every third day. On the day of the experiment, single-cell suspension will be prepared. The cells will be counted and the viability can be checked. The concentration can be adjusted with the medium containing 10% FBS and antibiotics.

2.4 Establishing cell cultures from frozen cells

About 10 ml of growth medium placed in a 15-ml conical tube. Thaw the frozen cryovial of cells for 40–60 s by gentle agitation in a 37°C water bath. Remove the cryovial from the water bath, and decontaminate the cryovial by immersing it in 70% (v/v) ethanol at room temperature (RT). Transfer the thawed cell suspension to the conical tube containing 10 ml of growth medium. Cells can be collected by centrifugation at 2000 rpm for 5 min at RT. Remove the growth medium by aspiration, and then resuspend the cells in the conical tube in 5 ml of fresh growth medium. Add 10 ml of growth medium to a 75-cm^2 tissue culture flask, and transfer 5 ml of cell suspension to the same. Place the cells in a 37°C incubator at 5% CO_2. Monitor cell density daily. Cells can be passaged when it will attain 50% confluency.

3. Determination of cell viability by trypan blue exclusion method

Trypan blue is a dye used to determine the viability of a cell. Living cells exclude the dye, and dead cells will take up the blue dye. The blue stain is easily visible, and cells can be counted using a light microscope. The reactivity is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells that exclude the dye are viable. When the cells are confluent, remove the cell media through aspiration, and add 5 ml of PBS swirl and aspirate. Then add 2 ml of trypsin/EDTA, and swirl to cover the monolayer of cells. Incubate for few minutes at 37°C. To remove the cells, strike the side of the plate or the flask with the palm. Check under a microscope to ensure that all the cells are dislodged. Add 8 ml of cell media containing fetal calf serum (FCS) to the cells containing the culture flask. The FCS will neutralize the action of trypsin. Transfer the cell suspension to a sterile centrifuge tube, and centrifuge the cell suspension at 1000*g* to pellet the cells. Wash the cell pellet twice with PBS. Resuspend the cell pellet in appropriate volume of PBS or cell media. Dilute 10 μl of cell suspension, and place 10 μl on a hemocytometer, and count the cells under a microscope. There are grid markings on the hemocytometer that can be seen under magnification. Count the cells in all four other quadrants of the grid. Divide this number by four to determine the average number of cells in one quadrant. To calculate the number of cells, multiply the average number of cells per quadrant by dilution factor. Multiply this number by 10,000 to calculate the number of cells in 1 ml of suspension. The equation is as follows: average number of cells per quadrant C dilution factor C 10,000 = number of cells/ml. To calculate the total number of cells, multiply the number of cells per ml by the volume (ml) of the cell suspension.

Calculating the % of viable cells: The cells (10,000) are suspended in 500 μl media and treated with varying concentrations of drug and incubate for 24 h. Centrifuge at 1500 rpm for 10 min. Discard 400 μl medium. Resuspended the pellet in the remaining medium. Mix 0.5 mg of trypan blue in 1 ml PBS. Take 10 μl of cell suspension and mix with trypan blue solution. Incubate for 5 min at room temperature. Count the numbers of unstained cells on the hemocytometer under a microscope. As mentioned above the dead cells will take up the trypan blue stain. First count the blue cells in the field and then white cells. Count the total number of

cells. The percentage of viable cells can be determined by dividing the number of unstained cells by the total number of cells and multiplying by 100. The equation is as follows: % of Cytotoxicity = [No. of blue cells/Total no. of cells] × 10.

3.1 Evaluation of cytotoxicity using MTT assay

The assay detects the living cells, and it be used to measure cytotoxicity, proliferation, or activation [18]. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay is based on the principle that mitochondrial dehydrogenase enzyme forms viable cells to cleave the tetrazolium rings of the pale yellow MTT and forms a dark blue formazan crystal which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The solubilization of the cells is by the addition of a detergent that cause in the liberation of the crystals, which are solubilized. The color can be quantified using a multi-well plate reader at 570 nm. The cells can be maintained in DMEM medium, supplement with 10% FCS. Briefly, cells in the log phase of growth can be harvested, counted, and seed (5×10^3 cells/well in 100 µl) in 96 well titer plates (Axygen), and add PBS to the outer wells (200 µl/well). After 24 h of incubation at 37°C in 5% CO_2 to allow cell attachment, media will be removed; cultures will be treated with various concentration of compounds diluted with medium. Cells and media are used as the negative controls. The plates are further incubated for 24, 48, and 72 h. On completion of incubation, with the extract, media can be removed without disturbing the adherent cells. In the case of suspension cells lines, the media can be removed after the plates are centrifuge at 2000 rpm for 15 min. To each well, 100 µl of 5 mg/ml stock solution of MTT dye will be added, and plates can be incubated for 2 h in the dark at 37°C in a CO_2 incubator. About 100 µl of lysis buffer can be added to each well, and the plates can be incubated for 4 h in the dark in a CO_2 incubator and absorbance can be read using ELISA plate reader. Three replicates are set up for each concentration. The concentration required to reduce absorbance by 50% (IC_{50}) in comparison to control cells is determined. In MTT assay colorless well indicates the cytotoxicity of KB human oral cancer cells (**Figure 2**)

$$1\ \%\ \text{of Growth inhibition} = 100 - \frac{\text{Absorbance of treated cells} \times 100}{\text{Absorbance of control cells}} \quad (1)$$

3.2 WST-1 assay

This method is used to detect the cytotoxicity of compound towards various cancer cell lines. The mitochondrial dehydrogenase enzyme cleaves the tetrazolium salt to formazan. The amount of the dye generated by the activity of

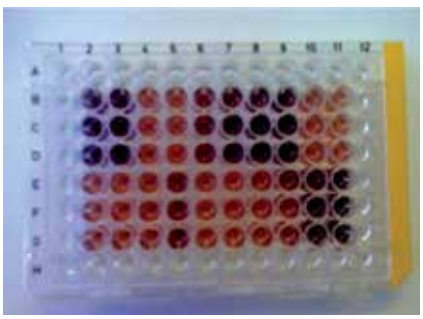

Figure 2.
MTT assay: purple color indicate the viability of cells.

dehydrogenase is directly proportional to the number of living cells. In the WST-1 assay protocol, add the WST-1 assay reagent to the cell culture media, and incubate for between 0.5 and 4 h, shake well. The formazan crystals produced by viable cells can be quantified and can be read in microplate reader at 440 nm. Cellular proliferation represents the ability of healthy cells to divide and create progeny [19]. Therefore, cell viability assays and cell proliferation assays are used to calculate the number of healthy cells in a population or the rate of growth of a population of cells [20].

3.3 MTS assay

MTS is a colorimetric method used to quantify viable cells in proliferation assay. The NAD(P)H-dependent dehydrogenase enzymes in metabolically active cells reduce the MTS tetrazolium compound and form colored formazan product that is soluble in cell culture media. It can be used to test cell proliferation, cell viability, and cytotoxicity. The formazan dye can be quantified by measuring the absorbance at 490–500 nm.

4. Viability assay in normal cells

Attempts are pursued to develop drugs that are nontoxic to normal cells; meanwhile toxic to cancer cells can be considered as good anticancer agent.

4.1 Isolation of lymphocytes from whole blood

In 1968, Boyum [21] described methods for the isolation of mononuclear cells from circulating blood and bone marrow. The solution contains Ficoll and sodium diatrizoate, adjusted to a density of 1.077 ± 0.001. This medium facilitates rapid recovery of viable lymphocytes from small volumes of blood on centrifugation. It is usually employed as the initial isolation step prior to enumeration of T, B, and null lymphocytes. In brief, 3 ml of blood will be taken in heparinized test tube. About 5 ml of PBS will be added and mixed well by inversion. About 3 ml of Ficoll Hypaque solution was added in a conical centrifuge tube. Carefully layered 8 ml of blood-PBS mixture on to the Ficoll Hypaque, keeping the tube in a slanting position. Centrifuged at 2000 rpm for 30 min. The opaque interface containing mononuclear cells was aspirated and transferred into a clean conical centrifuge tube with a Pasteur pipette and discard the upper layer. About 10 ml of PBS solution was added and mixed by inversion. Centrifuged at 1500 rpm for 10 min and the supernatant was discarded. Resuspend the pellet with 5 ml PBS and centrifuge at 1500 rpm for 10 min. Repeat the step thrice and resuspend the lymphocyte pellet in 500 μl PBS.

4.2 Lymphocyte viability assay

In vitro experiments with compounds can be tested in lymphocyte using lymphocyte viability assay. The lymphocytes were aspirated from the gradient plasma interfaces and washed twice in PBS, and then the final cell pellets will be resuspended in RPMI-1640 medium containing 10% FCS and 100 μl streptomycin, and 100 μl fungicide can be added to avoid contamination; pH 7.4 is ideal. Cells can be harvested, counted, and seeded (5×10^3 cells/well in 100 μl) in 96 well titer plates (Axygen). PBS will be added to the outer wells (200 μl/well). After 24 h of incubation at 37°C in 5% CO_2 to allow cell attachment, the media will be

removed. Cells will be treated with varying concentration of compounds diluted with medium. The plates can be incubated for 72 h. After incubation, the media will be removed without disturbing the cells, and to each well, 100 µl of 5 mg/ml stock solution of MTT will be added, and the plates can be incubated for 2 h in the dark at 37°C in a CO_2 incubator. About 100 µl of lysis buffer will be added to each well, and the plates can be further incubated for 4 h in the dark in a CO_2 incubator, and absorbance can be read using ELISA plate. Three replicates will set up for each concentration.

5. Detection of apoptosis by comet assay

Comet assay can be done for the quantitation of low levels of DNA damage in individual [22]. Cancer cells except the control cells can be treated with various drugs. Then it can be centrifuged to get the pellets, and pour 1% NMPA agar on the base slides using filler pipette and allow to solidify. Then the slides were kept in a polar ice pack. The pellets thus obtained will be taken and is mixed with 200 µl of 0.5% LMPA agar. About 15 0 µl cells from the above tubes were taken and poured over the base slides, and allow to solidify after placing a cover slip over it, and then remove the cover slip and pour a layer of 1% LMPA agar over it, and allow to solidify and keep these slides in a coupling jar containing lysing solution. It will be kept in refrigerator for 1:30 h, and subject it to electrophoresis for 20–30 min in electrophoresis apparatus at 25 V. Then the slides were taken and washed with neutralizing buffer solution for three times. Pour ethidium bromide stain over the slides, and view through fluorescent microscope. The figure shows the comet assay on KB human oral cancer cells after treatment with compound for 24 h (**Figure 3**).

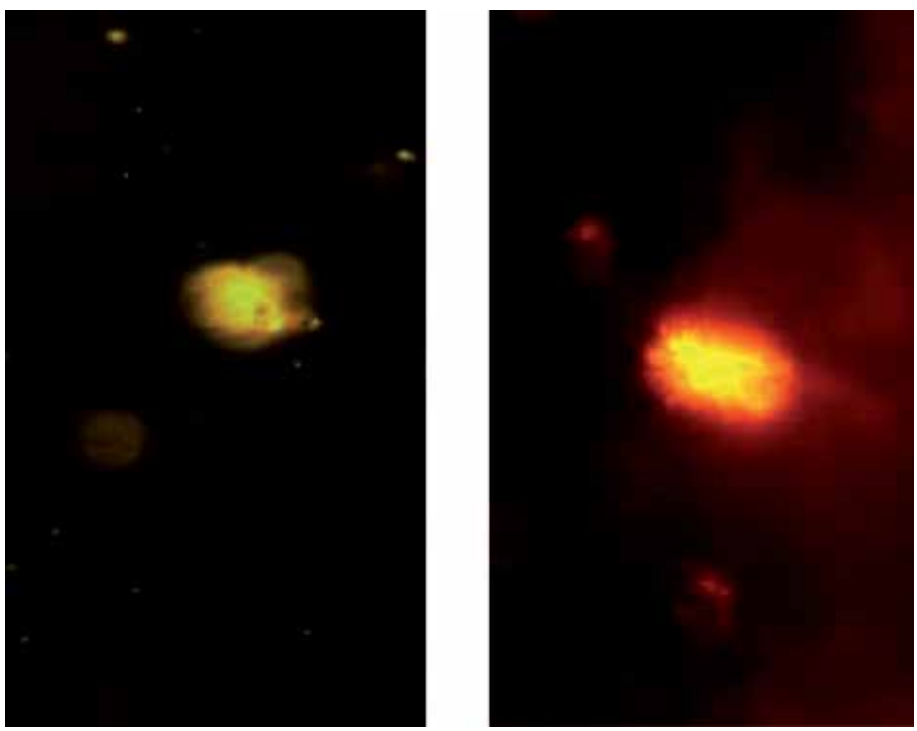

Figure 3.
Comet assay: (a) non-apoptotic cells, (b) comet-shaped apoptotic cells.

5.1 DNA ladder assay

Breakdown of genomic DNA into multiples of approximately 180 bp is considered to be a hallmark of apoptosis [14]. This cleavage of chromosomes produces a large number of DNA breaks, and subsequently a simultaneous amount of new 3′-OH DNA ends. In normal living cells, only an insignificant number of 3′-ends are present; this helps to detect apoptosis. The enzyme terminal deoxynucleotidyl transferase (TdT) has the capability to incorporate individual deoxyribonucleotide triphosphates to the 3′-end of double- or single-stranded DNA. This quality can be detected using 3′-ends with nucleotides that have been labeled with radioactive, fluorescent, or digoxigenin labels. Apoptosis can be measured quantitatively by using gel electrophoresis; here the apoptotic DNA is organized into a typical ladder pattern of multiples of 180 bp. In situ labeling of 3′-ends can be used to detect qualitatively apoptotic cells (**Figure 4**).

5.2 Detection of morphological features of apoptotic cells by acridine orange-ethidium bromide dual staining

The morphological features of apoptosis induced by the compounds will be evaluated by acridine orange-ethidium bromide dual (AO/EtBr) staining [23]. Briefly, cells

Figure 4.
Apoptotic cells can be seen as fragmented DNA in smear appearance.

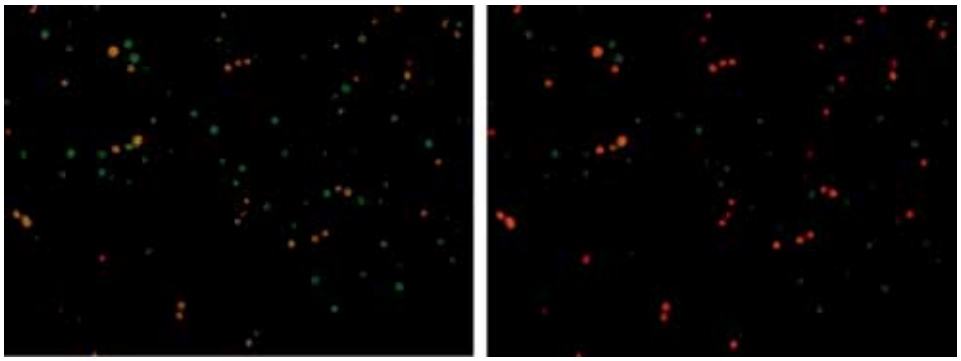

Figure 5.
Acridine orange-ethidium bromide dual staining: (a) and (b) apoptotic cells in orange color, live cells in green color.

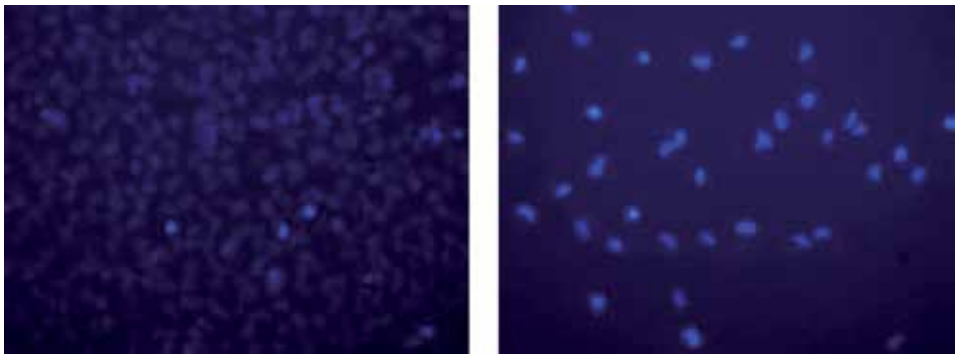

Figure 6.
Hoechst 33342: (a) non-apoptotic cells in blue color, (b) apoptosis cells in fluorescent blue color.

will be seeded in a 96 well plate at a density of 5×10^5 cells and treat with different concentrations of compound for 24 h. After washing once with PBS, the cells can be stained with 100 µl of a mixture (1:1) of acridine orange-ethidium bromide (4 µg/ml) solutions. The cells can be immediately washed with PBS and observed under fluorescence microscope at 450–490 nm. The effects of the compound treated on human oral cancer cells (KB) for 24 h can be visualize using fluorescent microscope (**Figure 5**).

5.3 Analysis of cell death by Hoechst 33342 staining

Chromatin condensation will be assessed by nuclear staining with Hoechst 33342 [24]. Briefly, cells will be seeded in a 96 well plate at a density of 5×10^5 cells and then treated with different concentrations of compounds for 24 h. After washing once with PBS, the cells will be stained with 100 µl of Hoechst 33342 (10 mg/ml stock) and incubate at room temperature for 5 min. Stained cells can be imaged by fluorescence microscope at 350–460 nm compound induced in KB human oral cancer cells for 24 h, stained with Hoechst 33342 (**Figure 6**).

5.4 Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

After treatment with the compound apoptosis, induction can be detected by TUNEL assay using the DeadEnd apoptosis detection kit (dUTP nick-end labeling

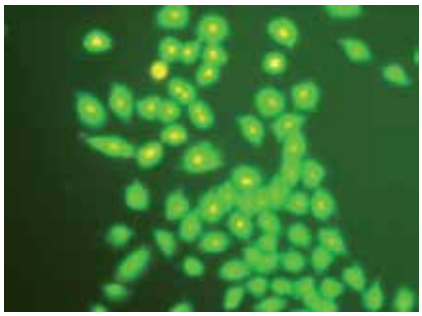

Figure 7.
TUNEL assay.

TUNEL assay) from Promega (Madison, USA). Briefly, the cells will be grown in coverslips and treated with the compound for 24 h. The cells can be washed with phosphate-buffered saline and fix by immersing the slides in 4% paraformaldehyde for 25 min at room temperature, washed twice with PBS for 5 min. The cells will be permeabilized with 0.2% Triton X-100 solution in PBS for 5 min, washed twice in PBS, and then covered with 100 μl of equilibration buffer and kept for 5–10 min. The equilibrated areas will be blotted around with tissue paper, and 100 μl of terminal deoxynucleotidyl transferase (Tdt) reaction mix will be added to the sections on the slide and incubated at 37°C for 60 min inside a humidified chamber for the end labeling reaction to occur. Immersing the slides in 2× sodium chloride-sodium citrate buffer for 15 min terminated the reactions. The slides will be washed thrice by immersing in fresh PBS for 5 min to remove the unincorporated biotinylated nucleotides. The endogenous peroxidase activity was blocked by immersing the slides in 0.3% hydrogen peroxide. After washing, horse radish peroxidase-labeled streptavidin solution was applied, and the slides incubated for 30 min. After incubation, the color will be developed with the peroxidase substrate (hydrogen peroxide) and the stable chromogen (diaminobenzidine). The slides will be mounted and examined in a light microscope. The apoptotic index (AI) can be calculated as follows: AI = (number of apoptotic cells/total number) × 100%. TUNEL can also be combined with annexin V to comprise a more robust assay that is capable of distinguishing apoptosis and necrosis going on in the cells. Since annexin V binding is reported to occur prior to DNA fragmentation, it is capable of detecting necrotic or early apoptotic cells that exhibit a negative response from TUNEL [25]. In KB human oral cancer cells treated with compound for 24 h, apoptotic cells can be seen in green color (**Figure 7**).

5.5 Annexin V staining by flow cytometry

In most normal, viable eukaryotic cells, the negatively charged phospholipid phosphatidylserine (PS) is located in the cytosolic leaflet of the plasma membrane lipid bilayer. Annexin V is a 36 KDa phospholipid-binding protein and has a high affinity to PS in the presence of physiological concentrations of calcium (Ca^{2+}). Apoptotic cells which are otherwise undetectable by staining with propidium iodide (PI) can be directly detected through the staining with fluorochrome-conjugated annexin V. Dead cells are stained with both annexin V and PI, whereas viable cells cannot be stained with either annexin V or PI. An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid. Phosphatidyl serine from the cytoplasmic interphase translocated to the extracellular surface. This loss of membrane asymmetry was detected using the binding properties of annexin V to phosphatidyl serine. Annexin V FITC is a sensitive probe for identifying the

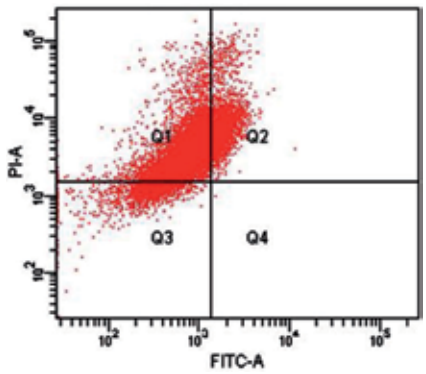

Figure 8.
Apoptotic KB human oral cancer cells after treatment with compound showing apoptosis.

apoptotic cells which binds to negatively charged phospholipid surfaces. Annexin V FITC staining precedes the loss of membrane integrity which accompanies the later stages of cell death resulting from apoptotic or rather necrotic process. Therefore, staining with annexin V FITC is conjugated to vital dye propidium iodide. The assay distinguishes between viable cells (annexin V –ve, PI –ve), early apoptotic cells (annexin +ve PI –ve), late apoptotic cells (annexin +ve PI +ve), and necrotic cells (annexin –ve PI +ve) (**Figure 8**). Annexin V is represented in red. An apoptotic cell stained with annexin V (green) is surrounded by potential phagocytes. The PSR (orange) might bind to exposed PS that is configured in a recognizable "eat-me" form by annexin I (bottom) (**Figure 8**). Alternatively, PSR might bind to a ligand composed of PS and annexin I (upper left) or PSR might bind annexin I, which serves as a bridge between exposed PS on the dying cell and PSR on the phagocyte (upper right) [26].

5.6 Assessment of caspase 3 expression by flow cytometry

Caspase-3 is a key protease that is activated during apoptosis. Briefly, the cells will be treated with various concentrations of compound. After 24 h of incubation, the cells will be washed twice with cold PBS and prepared for acquisition using FITC conjugated monoclonal active caspase-3 antibody apoptosis detection kit. The cells can be fixed in cytofix solution at a concentration of 1×10^6 cells/0.5 ml. The cells will be fixed in ice for 30 min, resuspended in perm wash buffer containing antibody, and incubated for 30 min at room temperature. Analyses by flow cytometry. 10,000 cells can be acquired, and the results can be interpreted using DIVA software analysis (**Figure 9**).

5.7 Determination of caspase activity by fluorimetry

The ApoAlert caspase assay plates contain the fluorogenic substrates specific for different caspases immobilized in the wells of a 96-well plate. When cell lysate containing the active caspase is applied to the wells, caspase will cleave its substrate, and a fluorescent product will be released that can be detected with a standard fluorescence plate reader. Caspase assay plates enable the analysis of the apoptotic caspase response. This assay design is ideal for studies involving multiple cell types or multiple cell treatments. These plates are provided in two formats: a single caspase format for studies that focus on a specific caspase or a profiling format for analyzing several different caspases simultaneously (caspase-3, caspase-8, caspase-9, and caspase-2).

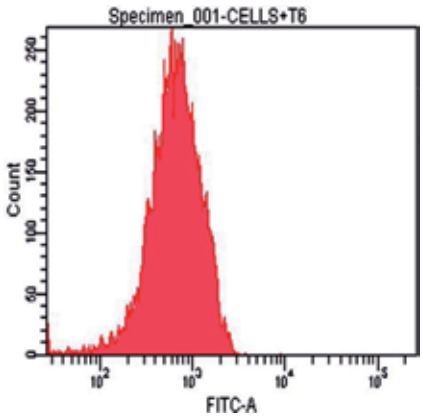

Figure 9.
Caspase 3 cleavage of KB human oral cancer cells.

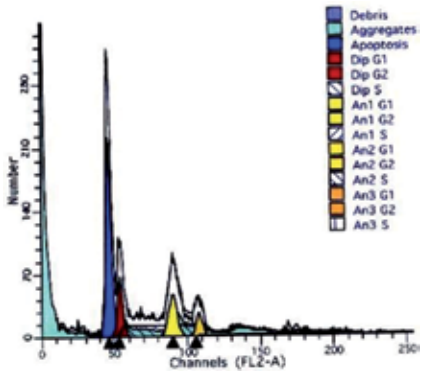

Figure 10.
Cell cycle analysis showing KB human oral cancer cells.

5.8 Detection of apoptosis by cell cycle analysis

The phase of the cell cycle at which compounds treated cancer cells got arrested can be determined using a fluorescent-activated cell sorter (FACS—Becton Dickinson). The cells will be grown in tissue culture flasks and treat with different concentrations of compounds. After 24 h of treatment, the cells can be harvested and spun down at 3500 rpm for 7 min. The cells can be then fixed in 70% ethanol for 30 min. After centrifugation, the pellet can be dissolved in PBS, and 5 μl of RNAse A (10 mg/ml) will be added and incubated for 30 min at 37°C. About 10 μg/ml of propidium iodide will be added, and after 15 min of incubation in the dark, the cells can filtered through 0.75 μm filter and analyzed by flow cytometry. Thirty thousand cells can be acquired, and the results can be interpreted using CellQuest Pro software analysis (**Figure 10**).

6. Conclusions

An ideal compound should possess no toxic effects on normal human lymphocyte but at the same time exhibited cytotoxic activity on tumor cell lines. The time- and dose-dependent cytotoxic effect of the compound can be tested through

the various viability assays discussed in this chapter. The compound might have the potential to induce programmed cell death in cancer cells and can be confirmed through apoptotic studies. Induction of apoptosis in cancer cells is recognized as an efficient strategy for cancer chemotherapy. Apoptosis also seems to be a reliable marker for the evaluation of potential agents to bring out for cancer prevention. Cell-based assays are useful for the assessment of live cells and apoptotic cells after treatment with therapeutic agents. The efficacy of compounds in vitro testing before entering into the clinical trials helps to bring out potent drugs into the limelight for the treatment of diseases. Cell-based assays are useful for evaluating therapeutic potency of the developing approved drugs and biologics for the clinical management of cancer.

Conflict of interest

There are no conflicts of interests.

Acronyms and abbreviations

AO/EtBr	acridine orange-ethidium bromide dual staining
KB	human oral cancer cell lines
KDa	kilodalton
FACS	Fluorescent-activated cell sorter

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References

[1] Godbey WT. In: Godbey WT, editor. An Introduction to Biotechnology. Woodhead Publishing; 2014. pp. 107-142. DOI: 10.1016/b978-1-907568-28-2.00005-8

[2] Holland P, Nielsen J, Mondeel TDGA, Barberis M. Coupling cell division to metabolic pathways through transcription. In: Ranganathan S, Gribskov M, Nakai K, Schönbach C, editors. Encyclopedia of Bioinformatics and Computational Biology. Oxford: Academic Press; 2019. p. 74-93. DOI: 10.1016/B978-0-12-809633-8.20081-2

[3] Paweletz N. Walther flemming: Pioneer of mitosis research. Nature Reviews Molecular Cell Biology. 2001;**2**:72-75. DOI: 10.1038/35048077. [Epub 2001/06/20]

[4] Lowe SW, Cepero E, Evan G. Intrinsic tumour suppression. Nature. 2004;**432**:307-315. DOI: 10.1038/nature03098. [Epub 2004/11/19]

[5] Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature. 2001;**411**:342-348. DOI: 10.1038/35077213. [Epub 2001/05/18]

[6] Kamsteeg M, Rutherford T, Sapi E, Hanczaruk B, Shahabi S, Flick M, et al. Phenoxodiol—An isoflavone analog—Induces apoptosis in chemoresistant ovarian cancer cells. Oncogene. 2003;**22**:2611-2620. [Epub 2003/05/06]. DOI: 10.1038/sj.onc.1206422

[7] Wyllie AH, Kerr JF, Currie AR. Cell death: The significance of apoptosis, International Review of Cytology. 1980;**68**:251-306. DOI: 10.1016/s0074-7696(08)62312-8. [Epub 1980/01/01]

[8] Ashkenazi A, Holland P, Eckhardt SG. Ligand-based targeting of apoptosis in cancer: The potential of recombinant human apoptosis ligand 2/tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). Journal of Clinical Oncology. 2008;**26**:3621-3630. DOI: 10.1200/JCO.2007.15.7198. [Epub 2008/07/22]

[9] Elmore S. Apoptosis: a review of programmed cell death. Toxicologic Pathology. 2007;**35**:495-516. DOI: 10.1080/01926230701320337. [Epub 2007/06/15]

[10] Lane D. Anthony Dipple carcinogenesis award. P53 from pathway to therapy. Carcinogenesis. 2004;**25**:1077-1081. DOI: 10.1093/carcin/bgh186. [Epub 2004/06/19]

[11] Bazzoni F, Beutler B. The tumor necrosis factor ligand and receptor families. The New England Journal of Medicine. 1996;**334**:1717-1725. DOI: 10.1056/NEJM199606273342607. [Epub 1996/06/27]

[12] Roy S, Nicholson DW. Cross-talk in cell death signaling. Journal of Experimental Medicine. 2000;**192**:F21-F25 [Epub 2000/10/18]

[13] Kerr JF, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. British Journal of Cancer. 1972;**26**:239-257. DOI: 10.1038/bjc.1972.33. [Epub 1972/08/01]

[14] Wyllie AH. What is apoptosis? Histopathology. 1986;**10**:995-998 [Epub 1986/09/01]

[15] Fey SJ, Wrzesinski K. Determination of drug toxicity using 3D spheroids constructed from an immortal human hepatocyte cell line. Toxicological Sciences. 2012;**127**:403-411. DOI: 10.1093/toxsci/kfs122. [Epub 2012/03/29]

[16] Breslin S, O'Driscoll L. Three-dimensional cell culture: The missing link in drug discovery. Drug Discovery

Today. 2013;**18**:240-249. DOI: 10.1016/j.drudis.2012.10.003. [Epub 2012/10/18]

[17] Choi SH, Kim YH, Hebisch M, Sliwinski C, Lee S, D'Avanzo C, et al. A three-dimensional human neural cell culture model of Alzheimer's disease. Nature. 2014;**515**:274-278. DOI: 10.1038/nature13800. [Epub 2014/10/14]

[18] Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods. 1983;**65**:55-63. DOI: 10.1016/0022-1759(83)90303-4. [Epub 1983/12/16]

[19] Yang N, Ray SD, Krafts K. In: Wexler P, editor. Encyclopedia of Toxicology. Oxford: Academic Press; 2014. pp. 761-765. DOI: 10.1016/b978-0-12-386454-3.00274-8

[20] Präbst K, Engelhardt H, Ringgeler S, Hübner H. Cell Viability Assays. Springer; 2017. pp. 1-17

[21] Boyum A. Isolation of leucocytes from human blood. A two-phase system for removal of red cells with methylcellulose as erythrocyte-aggregating agent. Scandinavian Journal of Clinical and Laboratory Investigation. 1968;**97**:9-29 [Epub 1968/01/01]

[22] Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research. 1988;**175**:184-191. DOI: 10.1016/0014-4827(88)90265-0. [Epub 1988/03/01]

[23] Kirsch-Volders M, Sofuni T, Aardema M, Albertini S, Eastmond D, Fenech M, et al. Report from the in vitro micronucleus assay working group. Mutation Research. 2003;**540**:153-163. DOI: 10.1016/j.mrgentox.2003.07.005. [Epub 2003/10/11]

[24] Porn-Ares MI, Chow SC, Slotte JP, Orrenius S. Induction of apoptosis and potentiation of TNF- and Fas-mediated apoptosis in U937 cells by the xanthogenate compound D609. Experimental Cell Research. 1997;**235**:48-54. DOI: 10.1006/excr.1997.3641. [Epub 1997/08/25]

[25] O'Brien IE, Reutelingsperger CP, Holdaway KM. Annexin-V and TUNEL use in monitoring the progression of apoptosis in plants. Cytometry. 1997;**29**:28-33 [Epub 1997/09/23]

[26] Fadok VA, Henson PM. Apoptosis: Giving phosphatidylserine recognition an assist—With a twist. Current Biology. 2003;**13**:R655-R657. DOI: 10.1016/s0960-9822(03)00575-x. [Epub 2003/08/23]

Edited by Biba Vikas and Michael Fasullo

This book on cell growth is the ideal resource for a scientist who wishes to learn more about cell growth topics. It provides information on plant growth hormones, kinetic studies on cell growth, growth of fungal cells and production, cell growth measurement, ion homeostasis response to nutrient deficiency stress in plants, intracellular lipid homeostasis in eukaryotes, and cell-based assays in cancer research. Each topic begins with a summary of the essential facts. Chapters were carefully edited to maintain consistent use of terminology and approach of covering topics in a uniform, systematic format.

Published in London, UK

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