



ORIGINAL ARTICLE

Changes in radical scavenging activity of normal, endoreduplicated and depolyploid root tip cells of *Allium cepa*



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Abstract The plant cell responds to abiotic stress conditions by adjusting its cellular metabolism and various defensive mechanisms. Cellular metabolism involves changes in the cell cycle, in which the cell undergoes repeated rounds of endocycles leading to polyploidization. Defense mechanisms such as role of antioxidants are a key to understand plant adaptation. The present work describes endoreduplication and radical scavenging activity as two different defense mechanisms adapted by plants for their survival under stress condition. The work describes linkage of these two processes with each other under abiotic stress. Endoreduplicated root tip cells of *Allium cepa* were depolyploidized by exogenous phytohormones. Further, free radical scavenging activity from normal, endoreduplicated and depolyploidized root tips cells was observed to understand the role of phytohormones. Elevated free radical scavenging potential was observed in endoreduplicated cells compared to normal and depolyploidized cells. Based on these results, it was concluded that endoreduplication and antioxidant pathways are linked with each other through phytohormonal activities. The concentration of auxin and cytokinin regulates the activity of ascorbate oxidase enzyme, which in turn maintains the concentration of AsA within the cell. AsA level directs the prolyl-hydroxylation process of cell division proteins in quiescent center cells either toward endoreduplication process or cell division process.

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Abbreviations: ASC, Ascorbate; AsA, ascorbic acid; BAP, benzyl-amino purine; GR, glutathione reductase; GSH, glutathione; NAA, naphthyl-acetic acid; ROS, reactive oxygen species.

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1. Introduction

The plant cell responds to stress conditions by adjusting its cellular metabolism and various defensive mechanisms (Breusegem and Dat, 2006). Cellular metabolism involves changes in the cell cycle, cell division, changes in the endomembrane system, vacuolization of cells and changes in the cell wall architecture which improves stress tolerance of

cells. Plants that have certain genes (for example, CCS52A) over-expressed and display endoreduplication often have a more rapid life cycle and improved stress tolerance than plants not displaying endoreduplication (Yves, 2007). Endopolyploidy has also been observed in tissues of plants that have been exposed to salinity. Catarino (1965, 1968) found that sea water treatments induced endoreduplication followed by cell enlargement in root cortex cells of *Lobularia maritima* and *Bryophyllum crenatum* (Ceccarelli et al., 2006) also found chromosome endoreduplication in root cortex cells of *Sorghum bicolor* cv. 610 plants that had been exposed to sub-lethal salinity level (150 mM NaCl) during their early development. This treatment gave the plants the capacity to grow and set seeds at a NaCl concentration of 300 mM (Amzallag et al., 1990). These observations suggest a stringent correlation between chromosome endoreduplication in certain tissues and salt adaptation. The perception of stress involves the activation of signaling cascades that results in a prolonged S-phase and delayed entry into mitosis, resulting in the formation of endoreduplicated cells (Kitsios and Doonan, 2011). This response can lead to reduced yield in crops, but it has a more general adaptive significance in that the resultant plant is more likely to survive. Plants under environmental stress, actively reduce their vegetative growth to conserve and redistribute resources and thus increase their chance of survival if the stress becomes severe (Skiryecz and Inze, 2010).

Various defensive mechanisms involve the role of antioxidants in plant adaptation during abiotic and biotic stresses (Burritt and MacKenzie, 2003; Dixon and Paiva, 1995; Vranova et al., 2002; Myung-Min et al., 2009). There is overwhelming evidence that many antioxidants play a key role in plant adaptation to both abiotic and biotic stresses (Burritt and MacKenzie, 2003; Dixon and Paiva, 1995; Vranova et al., 2002). Antioxidants are particularly important in the context of organic chemistry and biology; all living cells contain complex systems of antioxidant chemicals and enzymes to prevent chemical damage to the cell components by oxidation. Also, they can interfere with the oxidation process by reacting with the free radicals, chelating free catalytic metals and also by acting as oxygen scavengers (Buyukokuroglu et al., 2001). Plants typically produce a diverse group of antioxidants as a protective mechanism against oxidative compounds which are produced in response to various stresses and are known to have a damaging effect on membranes, organelles and macromolecules (Mittler, 2002; Noctor and Foyer, 1998; Smirnov, 1998). They possess capabilities to cope up with oxidative stress by scavenging ROS using antioxidants such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbic acid (AsA, reduced form), glutathione and flavonoids (Kwon et al., 2001). Among the various antioxidants Ascorbate acts as an important antioxidant in both enzymatic and non-enzymatic (reacting directly with hydroxyl radicals, superoxide, and singlet oxygen) reactions in plant cells. Some of the monodehydroascorbate (MDHA), oxidized form of ascorbate, is re-reduced by MDHAR using NAD(P)H, but the remainder undergoes spontaneous dismutation to AsA (reduced ascorbate) and dehydroascorbate (DHA, oxidized ascorbate). DHAR catalyzes the re-reduction of DHA to AsA with simultaneous oxidation of GSH to GSSG. Thus, DHAR, as well as MDHAR, is critical for protection of cellular components against oxidative injury (Asada, 1999).

Arrigoni's group has emphasized the role of ASC (Ascorbate) during cell division at the root meristem (Arrigoni, 1994). It is required for the progression of the G1 and G2 phases of the cell cycle (Liso et al., 1984). Previously, a number of observations indicated that ASC and its free radical stimulate root elongation and accelerate the quiescence-proliferation transit in onion roots (Hidalgo et al., 1991; De Cabo et al., 1993; Gonzalez-Reyes et al., 1994). AsA is required for prolyl-hydroxylation of protein(s) required for progression through the cell cycle is the best supported by published data (Vera et al., 1994; Ito et al., 1998; De Tullio et al., 1999). Inhibition of prolyl hydroxylation inhibits cell division in tobacco protoplasts (Cooper et al., 1994) and causes the majority of onion root cells to arrest at metaphase (De Tullio et al., 1999). AsA levels are generally found to be high in meristematic tissue and very low in zones with little active cell division such as the maize root quiescent center (Smirnov, 1996). In parallel, in non-dividing cells ascorbate oxidase activity and mRNA level are high whereas during the transition into active cell division, they both are quite low (Kerk and Feldman, 1995; Kato and Esaka, 1999). Furthermore, treatment with exogenous AsA enables cell-division competent cells to progress more rapidly from G1 to S phase (Citterio et al., 1994). Although it appears from these data that AsA is involved in plant cell cycle, the mechanism of this involvement has not been extensively studied. In the present study, interaction between endoreduplication and radical scavenging activity in adaptation of cells during abiotic stress is determined. How antioxidants are coordinated with growth and development of higher plants to maintain an appropriate dynamic homeostasis for stress tolerance and efficient survival is discussed.

2. Materials and methods

2.1. Development of roots from onion bulb

Onion bulbs of uniform size were selected and washed thoroughly with water after removal of dry leaves in order to reduce fungal contamination. Bulbs were kept in a coupling jar filled with water for 3 days at room temperature for the development of young roots.

2.2. Treatment of roots with endoreduplicating agent

Young roots of onion were treated with various concentrations of colchicine ranging from 2 μ M to 250 μ M. Roots were exposed to colchicine treatment for 120 h at room temperature. Morphological and cytological changes in root tips of *Allium cepa* were observed at an interval of 24 h.

2.3. Treatment of roots with phytohormones

After 120 h of colchicine treatment endoreduplicated root tips were obtained. These root tips were exogenously treated with NAA for 120 h. Similarly, endoreduplicated root tips were exogenously treated with BAP for 120 h. Changes in morphological and cytological characters (length and width of roots, cell and nucleus size of root tip cells) were observed at an interval of 24 h. Roots were treated with different concentrations of NAA or BAP (1 μ M, 50 μ M, 100 μ M, and 250 μ M) to study

the concentration dependent response of cells during growth and development of roots.

2.4. Microscopic observation for mitosis

Microscope slides were prepared from normal and treated root tip samples at an interval of 24 h. Samples were observed using Carl Zeiss Axiovision microscope after feulgen staining (Ohri et al., 1998). Cell and nucleus size of root tip cells were measured during late prophase using the imaging power of Carl Zeiss Axiovision 4 software.

2.5. Measurement of antioxidant activity

2.5.1. Sample preparation

Normal root tips (0.5 g) and treated root tips (0.5 g) were harvested at 24 h intervals. Three replicates of 0.5 g of root tips were preserved in methanol. They were crushed in methanol by mortar and pestle. These crushed samples were centrifuged at 2700g for 10 min at room temperature. Supernatant was collected and methanol was added to it to make up the final volume to 5 ml.

2.5.2. Microplate assay for radical scavenging activity

Radical scavenging activity from methanol extract of normal and treated root tip samples were determined by using a stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), following the method of Velazquez et al. (2003) with some alterations. The reaction mixture contained 1 mM DPPH (100 μ l) and 100 μ l methanol extract in each microplate well. Samples were taken in triplicate and the reaction mixture was incubated at room temperature for 2 min. The decrease in absorbance of DPPH due to methanol extract of root tips was measured at 517 nm using a microplate reader (Bio-Tek μ Quant, USA). Radical scavenging activity of the root tip extract was calculated as mM equivalent ascorbic acid per gram fresh weight of root tip with \pm standard deviation. The blank sample was prepared using methanol. Methanol was considered as blank and a range of different concentrations of ascorbic acid was used for the preparation of a standard curve.

3. Results

3.1. Radical scavenging activity in normal and endoreduplicated cells

Endoreduplication and radical scavenging activity are the two different defense mechanisms used by plants for survival. To study the interaction between endoreduplication and antioxidant activity, radical scavenging activity was measured from normal and endoreduplicated root tip cells of *Allium cepa*. Endoreduplicated cells were developed by using various concentrations of colchicine (2 μ M, 50 μ M, 100 μ M, 150 μ M, 200 μ M and 250 μ M). Colchicine treatment inhibited further growth of roots and formed C-tumor at root tips (Fig. 1), which contained endoreduplicated cells. Radical scavenging activity was measured from this endoreduplicated cells. antioxidant activity of endoreduplicated cells was higher than that of normal root tip cells (Fig. 5). This suggests that colchicine treatment increased antioxidant activity in root tip cells. All

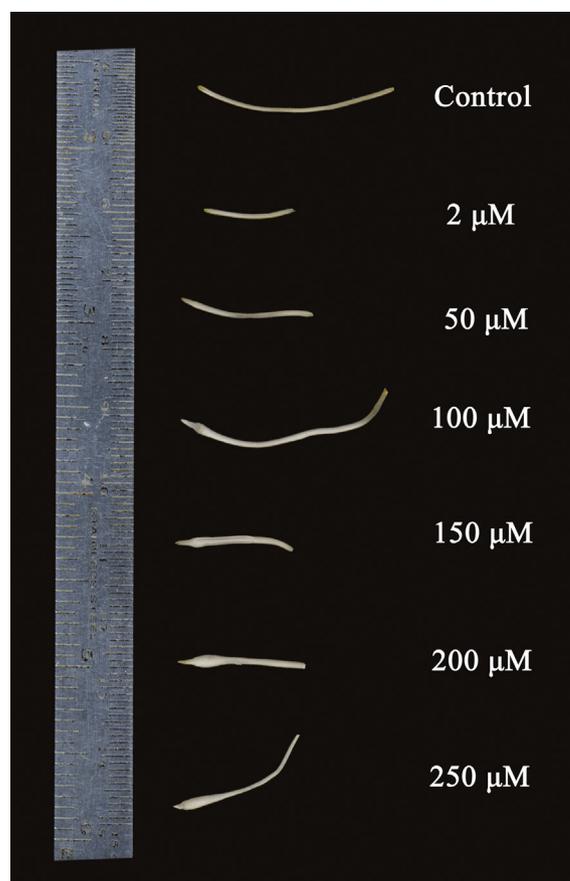


Figure 1 Changes in morphology of colchicine treated root tips after 24 h.

concentrations of colchicine tested showed higher antioxidant activity than the normal root tip cells. dose dependent response of colchicine concentration in increasing the antioxidant activity of root tip cells was observed. In lower concentrations (2 μ M, and 50 μ M) antioxidant activity was less compared to higher concentrations of colchicine (100 μ M, 150 μ M, 200 μ M and 250 μ M) tested (Fig. 5).

3.2. Radical scavenging activity in BAP treated endoreduplicated cells

Further, endoreduplicated roots were treated with different concentrations (1 μ M, 50 μ M, 100 μ M and 250 μ M) of phytohormone (NAA or BAP) to increase the growth of roots. However, only BAP gave positive response in increasing growth of roots (Fig. 2). It reduced the effect of colchicine and induced cell division and the differentiation process in roots (Fig. 4). Status of defense mechanism in BAP treated roots was studied by measuring radical scavenging activity and showed a decrease in antioxidant potential in all treatments of colchicine concentrations. It was observed that the antioxidant potential is remarkably lower in BAP treated root tip cells compared to endoreduplicated root tip cells. Its antioxidant activity was almost similar to that of the normal root tip cells (Figs. 6–8). This suggests that BAP reduced the defense mechanism in cells and diverted cell cycle from endoreduplication to cell division and differentiation process.

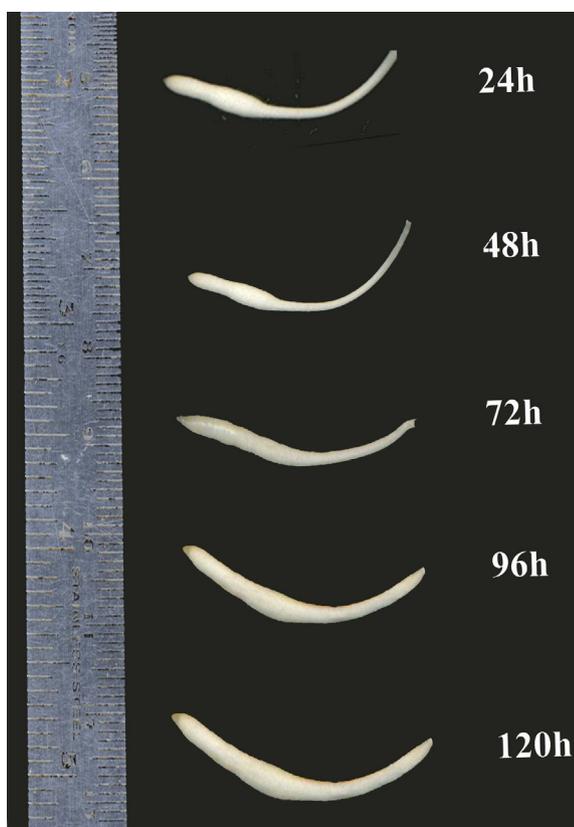


Figure 2 Changes in morphology of 250 µm colchicine treated root tips after 1 µM NAA treatment.

3.3. Radical scavenging activity in NAA treated endoreduplicated cells

Radical scavenging activity was measured from NAA treated root tip cells to know the status of defense mechanism in these cells, as, it was not able to increase the length of roots as well as not induce cell division and differentiation process in colchicine treated root tip cells (Figs. 3 and 4). In all concentrations of colchicine tested, antioxidant activity remained high after NAA treatment. Its antioxidant activity was almost similar to that of endoreduplicated root tip cells. Antioxidant activity in all concentrations of NAA (1–250 µM) treated cells was much higher than normal and BAP treated root tip cells (Figs. 6–8). This suggests that NAA was not able to reduce stress condition induced by colchicine and hence the defense mechanism in endoreduplicated cells remained constant.

4. Discussion

Root tip cells of *Allium cepa* were selected to study the function of endoreduplication in adaptation of plants against abiotic stress because the root apical meristem plays a fundamental role acting as sink and source of signals. They regulate the root system architecture and modulate the adaptation to environmental stress (Lynch, 1995). When meristematic cells of *Allium cepa* root tips were treated with various concentrations of colchicine, they had undergone endoreduplication to survive in response to abiotic stress created by colchicine.

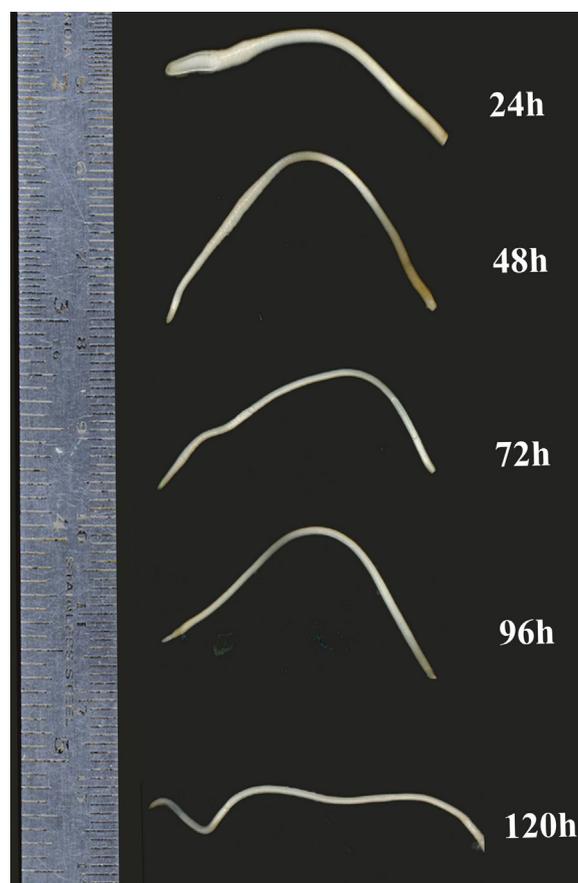


Figure 3 Changes in Morphology of 250 µm colchicine treated root tips after 1 µM BAP treatment.

Radical scavenging activity was measured from these endoreduplicated cells using ascorbic acid (AsA) as standard to know the role of endoreduplication in defense mechanism.

Antioxidants influence higher plant growth and development by modifying processes from mitosis and cell elongation to senescence and death. One of the key responses to moderate stress is the inhibition of cell proliferation and cell growth. This response can lead to reduced yield in crops, which has an adaptive significance in that plant and is more likely to survive (Kitsios and Doonan, 2011). Similar response was observed in roots of *Allium cepa* when they were treated with different concentrations of colchicine. There was inhibition of root growth and C-tumor formation was observed in root tips. Antioxidant activity remained high in these colchicine treated cells (endoreduplicated cells). The manipulation of the antioxidant system in the plant through overexpression of enzymes such as glutathione reductase (GR) leads to an increase in pool of ascorbate and GSH (Glutathione), known as small antioxidant molecules which are involved in ROS (Reactive Oxygen Species) scavenging and can maintain or improve the plant productivities under environment stress condition. When subjected to environmental stress, plants actively reduce their vegetative growth to conserve and redistribute resources and thus increase their chance of survival if the stress becomes severe (Skirycz and Inze, 2010). However, when the stress does not threaten survival, growth inhibition is counter-productive because it leads to an unnecessary drop in produc-

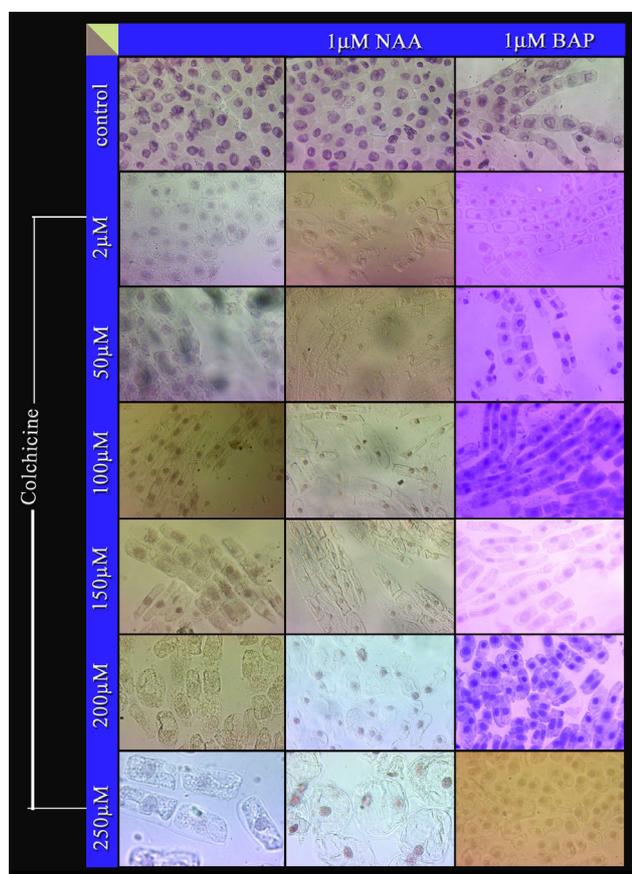


Figure 4 Changes in cell and nucleus size of colchicine treated root tip cells after 1 μM NAA and 1 μM BAP treatments.

tivity and substantial yield penalties. Gene Expression data have also revealed a down-regulation of cell cycle related transcripts only 24 h after stress imposition and intriguingly negative and positive cell cycle regulators were down regulated to the same extent (Skirycz et al., 2011; Tank and Thaker, 2014).

Among the various antioxidants, ascorbic acid was selected as standard because Regulation of AsA levels in root meristems appears to involve turnover through the activity of ascorbate oxidase. The down regulation of AsA in the non-dividing quiescent center of the root meristem is thought to involve an inter play between ascorbate oxidase and auxin. Accumulation of auxin in root tips results in increased levels of ascorbate oxidase and decreased AsA (Kerk and Feldman, 1994, 1995). Later Kerk et al., 2000 had proposed that ascorbate oxidase induced by auxin controls the depletion of AsA in the quiescent center of maize root tips. Ascorbate oxidase was shown to inactivate auxin by oxidative decarboxylation *in vitro*, and auxin is also decarboxylated by intact root tips (a major site of ascorbate oxidase) (Kerk et al., 2000). The transport of high levels of auxin to the root tip leads to an increase in ascorbate oxidase and a subsequent decrease in AsA, resulting in cell division inhibition. Similar results were obtained in the present studies, which showed that normal root tip cells when treated with NAA resulted in inhibition of root growth and cell division in root tip cells. Interestingly, it was also observed that there was no further growth of endoreduplicated roots as well as no initiation of cell division in endoreduplicated cells after NAA treatment. This suggests that decarboxylation of auxin by ascorbate oxidase permits control of root development and therefore regulation of quiescent center maintenance (Kerk et al., 2000). Auxins and cytokinins interact in the control of many central developmental processes in plants, particularly in apical dominance and root and shoot development (Nordstrom et al., 2004). Auxin triggers organogenesis and that Cytokinin modulates its output through its effect on auxin distribution, which is realized by Cytokinin dependent regulation of expression of auxin transport components (Pernisova et al., 2009). Differential auxin distribution has been shown to mediate multiple aspects of plant development, such as apical/basal axis formation (Friml et al., 2003), root patterning (Friml et al., 2002a; Blilou et al., 2005), tropisms (Friml et al., 2002b; Luschnig et al., 1998; Marchant et al., 1999), and organogenesis (Heisler et al., 2005; Benkova et al., 2003;

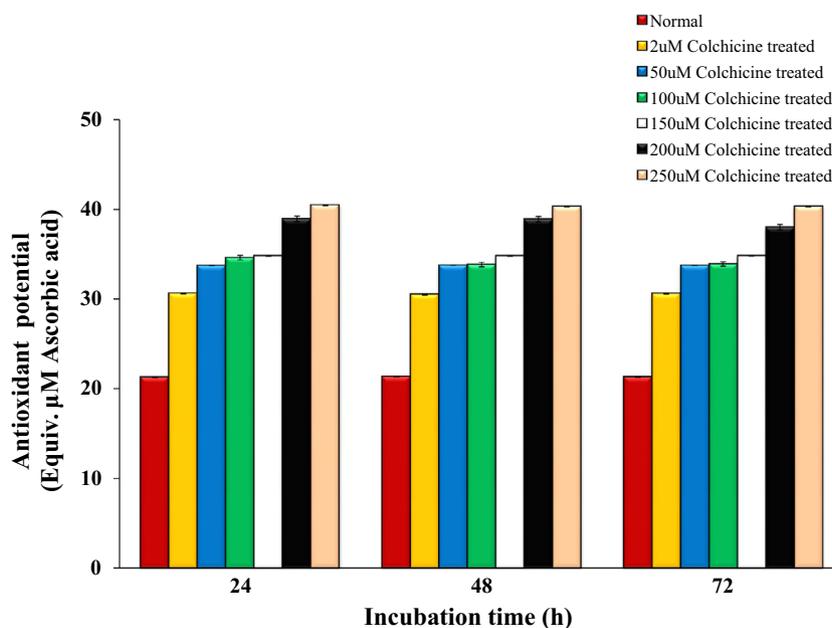


Figure 5 Antioxidant activity in colchicine treated root tip cells.

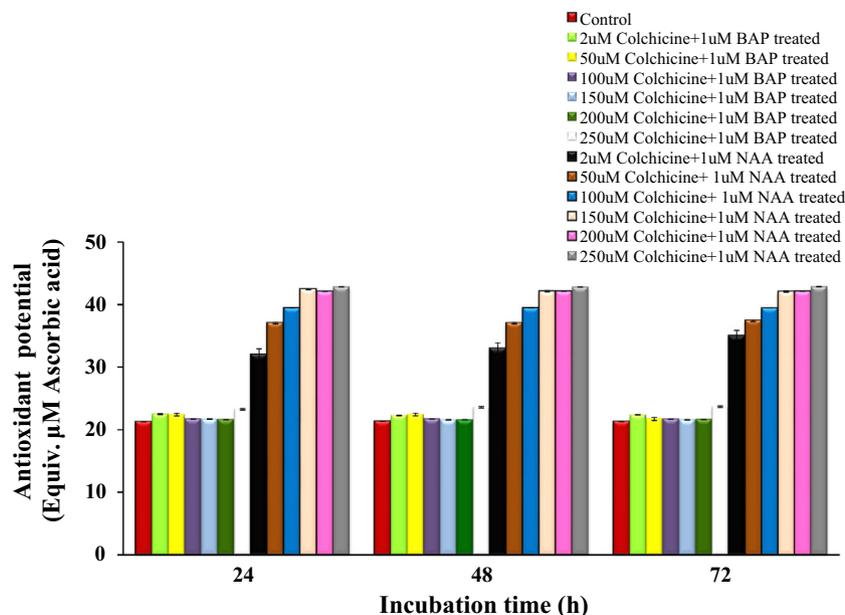


Figure 6 Antioxidant activity in endoreduplicated root tips cells after 1 μ M NAA and 1 μ M BAP treatments.

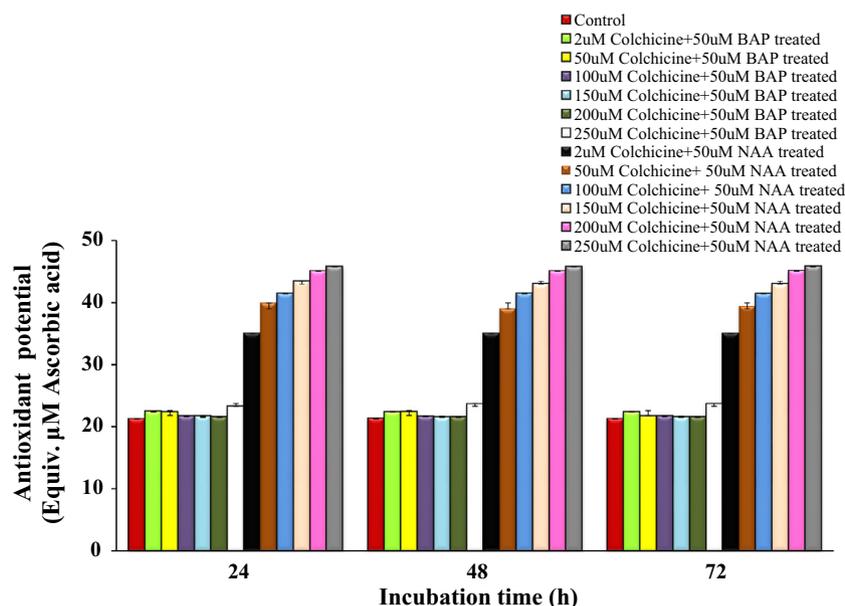


Figure 7 Antioxidant activity in endoreduplicated root tips cells after 50 μ M NAA and 50 μ M BAP treatments.

Reinhardt et al., 2003; Dubrovsky et al., 2008). Cytokinin is an important regulator of shoot (Kurakawa et al., 2007) and root architecture (Scheres et al., 1995; Mahonan et al., 2006; Werner et al., 2003; Laplaze et al., 2007; Ioio et al., 2007; Kuderová et al., 2008) and it also regulates seed development (Riefler et al., 2006), abiotic stress (Tran et al., 2007), and plant senescence (Kim et al., 2006). Cytokinin modifies the abundance of transcripts for several putative auxin biosynthetic genes, suggesting a direct induction of auxin biosynthesis by cytokinin. Cytokinin is essential, not only to maintain basal levels of auxin biosynthesis in developing root and shoot tissues but also for the dynamic regulation of auxin biosynthesis in response to changing developmental or environmental con-

ditions. Cytokinin-overproducing tobacco plants have been shown to contain lower levels of free indole-3-acetic acid (IAA) and reduced rates of IAA synthesis and turnover (Eklof et al., 1997, 2000). Similarly, in the present studies, we observed that BAP treatment to endoreduplicated cells induced cell division and differentiation process in root tip cells. It further increased length of roots by diverting the cell cycle toward cell division to differentiation process (Tank and Thaker, 2014). Antioxidant activity measured from these cells was much lower than endoreduplicated root tip cells of *Allium cepa*, but its antioxidant activity was almost similar as that of normal root tip cells of *Allium cepa*. In contrast, NAA treatment to endoreduplicated roots showed browning

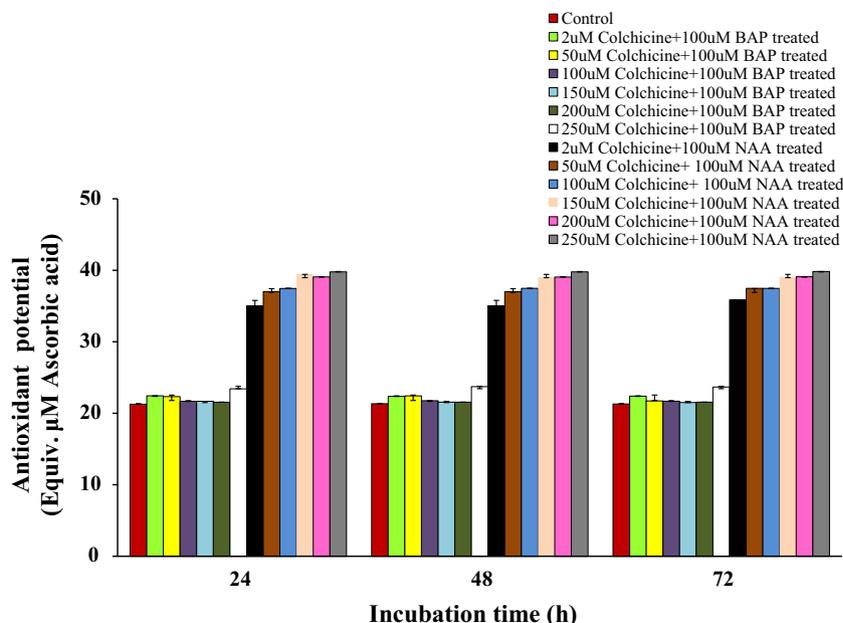


Figure 8 Antioxidant activity in endoreduplicated root tips cells after 100 μM NAA and 100 μM BAP treatments.

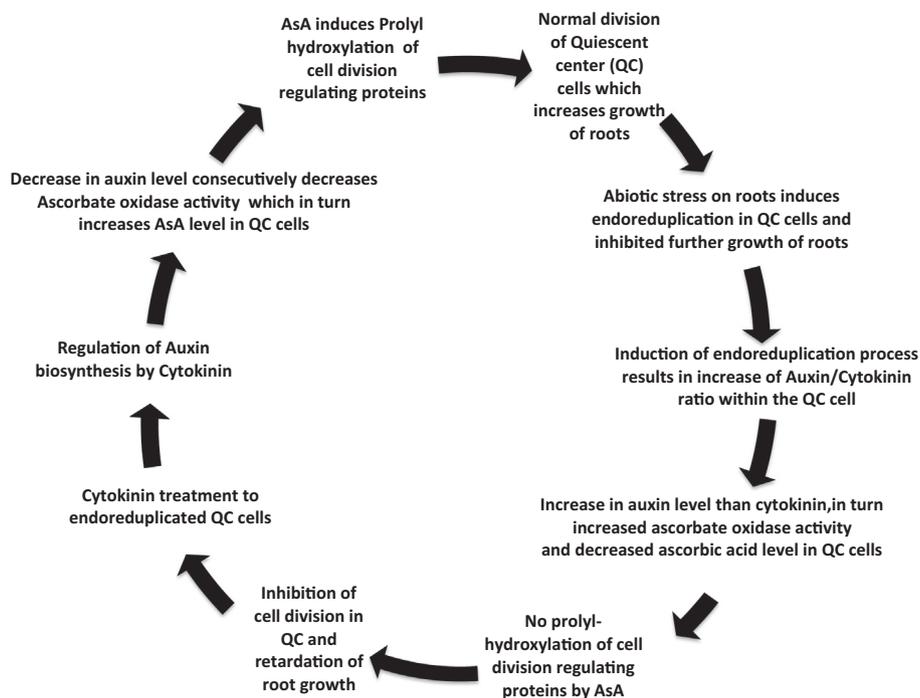


Figure 9 The pathway of metabolic mechanism taking place in quiescent center (QC) cells during stress. When a normal root quiescent center cell experiences abiotic stress from colchicine, it undergoes endoreduplication process due to increase in auxin/cytokinin ratio. Increased auxin/cytokinin ratio in turn increases the activity of ascorbate oxidase and decreases the level of ascorbic acid in quiescent center cells. Due to decrease in ascorbic acid level prolyl-hydroxylation of cell division regulating proteins does not take place. This results in inhibition of further growth and development of roots. When exogenous cytokinin treatment was given to these roots metabolic changes occurred in the endoreduplicated quiescent center cells. Cytokinin regulated auxin synthesis pathway and decreased auxin level in endoreduplicated quiescent center cells. This in turn decreased the ascorbate oxidase activity in quiescent center cells and increased the level of ascorbic acid within the cell. Ascorbic acid (AsA) provoked the prolyl-hydroxylation of cell division regulating proteins which again stimulated growth and development in roots.

and degradation of root tip cells. It diverted cell cycle toward senescence and death. Antioxidant activity measured in these cells was similar to that of endoreduplicated cells. This suggests that BAP reduced the stress condition in root cells and diverted cell cycle toward cell division and differentiation process.

5. Conclusion

From the above studies it was concluded that endoreduplication and antioxidant pathways are indirectly interlinked with each other through phytohormonal activities. It was observed that the concentration of auxin and cytokinin regulates the activity of ascorbate oxidase enzyme, which in turn maintains the concentration of AsA within the cell. AsA level controls the prolyl-hydroxylation process of cell division proteins. When AsA level increases within quiescent center cells then the cell cycle shifts toward normal cell division. However, when AsA level decreases in quiescent center cells then cell cycle shifts toward endoreduplication process (Fig. 9).

Acknowledgments

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