Cytotoxicity of different concentrations of oxalic acid on onion cells

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Abstract: The present study aimed to explore the effect of oxalic acid (OA) different concentrations (1, 2 and 4 g/L) and Sclerotium cepivorum crude extract on the cytology of Allium cepa germinating roots during four durations 6, 12, 18 and 24 hrs. The rate of cell division and mitotic chromosomal behavior in root tips of germinated bulbs beside the numbers and types of chromosomal abnormalities were recorded for the selected samples during the period of treatments after 6, 12, 18, 24 hrs by oxalic acid 1, 2, 4 g, and S. cepivorum crude extract. The results showed that the mitotic indices were reduced when the roots were treated by different concentrations of oxalic acid and S. cepivorum crude extract, and the reductions are proportional with both concentration and time. Also, several chromosomal abnormalities were recorded that includes vacuolated nuclei, rod-shape, binucleated cells, disrupted nuclei at interphase and lagged chromosomes, bridge, polyploidy, mega cell, star metaphase, mini-chromosomes, ring chromosomes, and deformed nuclei during mitosis. Results suggested that the oxalic acid affects pH value, Ca²⁺ depletion and plasma membrane maceration, and it induces programmed cell death in onion.

Keywords: Mitotic activities, Allium cepa, Sclerotium cepivorum, Oxalic acid, Chromosomal abnormalities, Mitotic Index.

Introduction

Onion (Allium cepa L.) is an important horticultural crop in Egypt. According to the latest report issued by the Agricultural Export Council, Egypt's exports of fresh onions reached to 302,549 tons during the export season 2020/2021. The onion crop is affected by many fungal and bacterial diseases at different growth stages which result in considerable losses in yield. For several years, the ways of controlling diseases were dependent on excessive use of chemicals that cause various environmental and health related problems and deleterious effects on plant growth, development, and other metabolic activities (Sengupta et al., 1989; Pankratz et al., 2003). Also, some chemicals might undergo various changes and become more toxic, mutagenic and affect plants, animals and human (Grigorenko & Larchenko, 2000; Bolognesi 2003; Amaroli et al., 2013). Due to that, research now focus on understanding the mechanisms of disease and knowing all about the interactions that occur between the causative agent of the disease and the host (Pathakumari et al., 2020).

One of the main and most danger fungal disease which affects the onion crop and causes yield loss up to 100% is Allium White Rot disease worldwide and in Egypt (Yousef, 2013), which caused by the soil-borne fungus S. cepivorum Berk. which produce small sclerotia serve as both propagules and inoculum due to the absence of a recognizable teleomorphic state (Couch & Kohn, 2000). These sclerotia can live and survive in the soil for more than 20 years and the germination can be occurred in response to the presence of onion sulfides to produce an infective mycelium of S. cepivorum (Coly-Smith et al., 1990). S. cepivorum can penetrate onion stem and grow inter and intracellular parenchyma causing cortical tissue disintegration and maceration of vascular tissue which cause a rapid watery rot of onion bulb scale (Abd-El-Razik et al., 1973). According to Bateman (1964), the maceration of host tissue by fungi depends on the fungal production of cell wall degrading enzymes, but in case of onion infection by S. cepivorum, oxalic acid is an essential factor beside the polygalacturonases to produce successful pathogenesis (Kritzman & Henis, 1977). The maceration of onion due to the infection by S. cepivorum is conducted by the synergistic action of endopolygalacturonase and oxalic acid (Stone & Armentrout, 1985).
This study aims to determine the effect of different concentrations of oxalic acid on onion root cells during different exposure periods versus the effect of *S. cepivorum* Berk. exo-secretion extract during the same exposure periods.

**Material and Methods**

*Allium Cepa* Giza 6 bulbs were purchased from The National Center for Agricultural Research, Dokki, Giza, Egypt. *S. cepivorum* identified isolate was obtained from Plant Pathology Department, Faculty of Agriculture, Mansoura University, Egypt. Isolate was re-germinated on modified onion root potato dextrose (PD) liquid media by supporting the PD broth with 50% per weight of onion roots instead of potato to induce the production of exo-secretion and incubated at 16°C for 12 days in dark condition. Fungal extract was obtained by filtration to remove the mate of fungus and the filtrate was used as crude extract.

For cytological studies, selected bulbs were surface sterilized and grown in sterilized distilled water for 4 days. Then, the bulbs were transferred into 1, 2, and 4 g/L of oxalic acid solutions, and a crude extract solution of *S. cepivorum* solutions for 6, 12, 18 and 24 hrs. Cytological preparations were carried out according to Hiremath and Chinnappa (2015) by fixation of excised roots from each treatment in Carnoy’s fixing solution of a mixture of glacial acetic acid and ethanol (1:3) for 2h. The fixed roots were hydrolyzed in 1N HCl for 8 min at 60°C, then washed thoroughly with distilled water and moved to Feulgen staining solution for 10 min. at room temperature in dark condition until the tissue stains deep purple. the stained root tips were then cut and squashed separately on clean glass slides in a drop of 50% glacial acetic acid solution and covered gently with clean covered slip and sealed with nail polisher. The slide visualization and cell count were done and photographed using Tucsen TCC-6.1CE 6.1 Megapixel 12-bit Cooled Color CCD Microscope Camera.

Several cytological parameters were calculated from 10 different slides for each treatment (approximately 500 cells per slide) by the following equations:

**Mitotic index (MI)** = (number of divided cells)/ (number of total counted cells) ×100.

**Phase index** = (number of divided cells per phase)/ (number of total counted cells) ×100.

**Relative Division Ratio (RDR)** = (MI treatment-MI control)/ (100-MI control) ×100.

**Relative abnormality** = (number of abnormal cells) / (number of divided cells) ×100.

**Absolute abnormality** = (number of abnormal cells) / (number of total counted cells) ×100.

Chromosomal aberrations were recorded as vacuolated cell, rod-shaped, disrupted nucleus prophase, budded nucleus, binucleated, mega cell, star metaphase, lagging metaphase, lagging anaphase, bridge anaphase, polyploidy, lagging telophase and bridge telophase, and represented as percentage of dividing cells. All experiments were recited thrice, and the software SPSS was used for statistical analysis.

**Results**

The rate of cell division, mitotic chromosomal behavior in root tips of germinated bulbs and the numbers and types of chromosomal abnormalities were recorded for the selected samples during the period of treatments after 6, 12, 18, 24 hrs by oxalic acid 1, 2, and 4 g/L and *S. cepivorum* crude extract (Table 1, Fig. 1, 2).

The results after 6 h of treatments revealed that mitotic index of control roots has the highest value (20.3%), and the MI were reduced to 13.5, 10, 0.4, and 8% when the roots treated by 1, 2, 4 g OA and *S. cepivorum* crude extract, respectively. The RDR were decreased to 8.4, 12.9, 14 and 24.9%, respectively. The interphase normal cells decreased from 86.4% in control to 81.1%, 78.5%, and 73.9%, and 12.9%, respectively.

Chromosomal Aberrations recorded in the interphase in this period were: vacuolated nuclei (9.2, 18, 18.6, 0.4 and 20.0%), rod-shape cell (4.4, 2.0, 6.3, 0.0 and 3.0%), binucleated cells (0.0, 0.5, 1.2, 19.6 and 4.4%) in of interphase cells in control, 1, 2, 4 g of oxalic acid and *S. cepivorum* crude extract, respectively. The disrupted nuclei were recorded only in the roots treated by *S. cepivorum* crude extract (60.7% of interphase cells).

The chromosomal aberrations in the interphase cells by the end of the first period were: 13.6, 18.9, 21.5, 26.1 and 87.1%, respectively. The prophase indices were 13.5, 8.3, 6.2 and 0.3%, respectively, the metaphase indices were 2.0, 1.7, 1.7, 0.2 and 0.1%, respectively, the anaphase indices were 1.4, 0.9, 0.9, 0.0 and 0.1%, respectively, the telophase indices scored 3.4, 2.6, 1.4, 0.0 and 0.0%, respectively.

The mitotic abnormalities recorded lagging metaphase (2.8, 9.2, 14.6, 20.0 and 14.6%, respectively), lagging anaphase (0.0, 2.3, 1.8, 0.0 and 26.8%, respectively), bridge anaphase (3.8, 2.1, 6.6, 0.0 and 19.5%), polyploidy (0.0, 0.5, 2.0, 0.0, 0.0%), lagging telophase (0.0, 17.4, 13, 0.0 and 0.0%, respectively), mega cell (up to 7.3% at 2 g OA), star metaphase (30% at 4 g OA), and bridge telophas (0.5% at control only), and the percentages of mitotic aberrations were 9.3, 33.7, 43.3, 50.0 and 61.0%, respectively.

After 12 hrs of treatments, mitotic index of control roots had the highest value (21.6%), and the MI were reduced to 18.4%, 5.3%, and 4.8% when the roots treated by 1, 2 and 4 g/L of oxalic acid, respectively. The lowest
value of MI was 0.2% which recorded in the roots that treated by *S. cepivorum* crude extract. The RDR were decreased to 4, 20.7, 21.4 and 27.3, respectively. The interphase normal cells decreased from 87.3% in control to 77.4%, 75.4%, and 49.6%, and 38.8%, respectively. Chromosomal Aberrations recorded in the interphase in this period were: vacuolated nuclei (8.3, 19.8, 20.35, 1.8 and 8.2%), rod-shape cell (3.2, 1.6, 0.0 and 3%), binucleated cells (0.0, 1.7, 28.0, 19.2 and 7.3%), and disturbed nuclei were recorded only in the roots treated by *S. cepivorum* crude extract (41.9% of interphase cells).

The chromosomal aberrations in the interphase cells by the end of the second period were 12.7, 24.6, 50.4, 22.6 and 50.4% at control, 1, 2, 4 g OA and *S. cepivorum* crude extract, respectively. The prophase indices were 14.2, 13.7, 3.0, 1.9 and 0.0%, respectively. The metaphase indices were 2.2, 1.9, 0.5, 1.6 and 0.2%, respectively. The anaphase indices were 1.4, 0.8, 0.9, 0.5 and 0.0%, respectively. The telophase indices scored 3.7, 2.0, 1.0, 0.7 and 0.0%, respectively.

The mitotic abnormalities noticed in all treated include lagging metaphase (3.4, 10.3, 9.0, 33.9 and 100%, respectively), lagging anaphase (0.0, 0.6, 0.0, 0.0 and 0.0%, respectively), bridge anaphase (2.6, 2.1, 12.6, 10.4 and 0.0%), polyploidy (0.9, 1.9, 4.7, 0.0, 0.0%), lagging telophase (0.0, 9.9, 18.0, 15.1 and 0.0%, respectively), megakaryocyte (up to 1.6% at controlled roots), star metaphase (0.0% at control and treated roots), and bridge telophase (0.5% at control only). The percentages of Mitotic aberrations achieved were 9.1, 24.7, 44.2, 59.4 and 100%, respectively.

The results after 24 hrs of treatments revealed that; Mitotic index of control roots has the highest value (20.1%), and the MI were reduced to 9.4%, 6.3%, and 6.6% when the roots treated by 1, 2 and 4 g of oxalic acid, respectively, the lowest value of MI was 0.0% which recorded in the roots that treated by *S. cepivorum* crude extract. The RDR were decreased to -13.3%, -17.2%, -16.8% and -25.1%, respectively. The interphase normal cells decreased from 86.4% in control to 78.9%, 28.5%, 68.7%, and 27.1%, respectively. Chromosomal Aberrations recorded in the interphase in this period were: vacuolated nuclei (9.2, 13.1, 18.7, 9.7 and 0%), rod-shape cell (4.4, 3.3, 4.1, 2.9 and 0.0%), binucleated cells (0.0, 4.6, 18.7, 18.6 and 2.1%) in of interphase cells in control, 1, 2, 4 g of oxalic acid and *S. cepivorum* crude extract, respectively. The disrupted nuclei were recorded only in the roots treated by *S. cepivorum* crude extract (70% of interphase cells). The chromosomal aberrations in the interphase cells by the end of the fourth period were 13.6, 21.1, 71.5, 31.3 and 72.9% at control, 1, 2, 4 g OA and *S. cepivorum* crude extract, respectively.

The prophase indices were 13.5, 7, 2.9, 3.4 and 0.0%, respectively. The metaphase indices were 2.0, 13.1, 1.9, 1.4 and 0.0%, respectively. The anaphase indices were 1.2, 0.2, 0.5, 0.9 and 0.0%, respectively. The telophase indices scored 3.4, 0.9, 1.0, 1.0 and 0.0%, respectively. The mitotic abnormalities noticed in all treated include lagging metaphase (2.6, 13.5, 30.8, 20.5 and 0.0%, respectively), lagging anaphase (0.0, 2.4, 0.0, 5.9 and 0.0%, respectively), bridge anaphase (3.4, 0.0, 7.7, 7.9 and 0.0%), polyploidy (up to 0.4% in controlled roots), lagging telophase (0.0, 10.1, 15.4, 14.7 and 0.0%, respectively), megakaryocyte (1.4, 24.4, 3.1, 2.6 and 0.0%, respectively), and bridge telophase (0.4% at control only). The percentages of mitotic aberrations recorded were 8.1, 50.3, 56.9, 51.6 and 0.0%, respectively.
| Table 1: Chromosomal aberrations in root tips of *Allium cepa* induced by 1, 2, and 4 g/L of oxalic acid, and *Sclerotium cepivorum* crude extract at 6, 12, 18, 24 h exposure periods |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Interphase       | Mitotic Division | Mitotic Aberrations | % Absolute Abnormality | % Relative Abnormality | Mitotic Index |
| Time            | Total Control Cell | Normal Cells | Abnormal Cells | Binucleated Cells | Bridge Cells | Missing Metaphase |
| %               | %               | 0 %          | 0 %             | 0 %             | 0 %          | 0 %              |
| Control         | 5283            | 4213         | 3639          | 389           | 185          | 0 %               | 0 %          |
| Ox 1 gm %       | 5200            | 4497         | 3649          | 810           | 15           | 0 %               | 0 %          |
| Ox 2 gm %       | 5480            | 4933         | 3647          | 916           | 310          | 0 %               | 0 %          |
| Ox 4 gm %       | 5165            | 5407         | 1008          | 0             | 0            | 0 %               | 0 %          |
| Sc. Cep. %      | 5062            | 5021         | 650           | 1003          | 98           | 3050             | 0            |
| %               | 87.1            | 88.7         | 87.3          | 87.6          | 83.7         | 96.8             | 80.8         |
| %               | 87.1            | 88.7         | 87.3          | 87.6          | 83.7         | 96.8             | 80.8         |
| %               | 87.1            | 88.7         | 87.3          | 87.6          | 83.7         | 96.8             | 80.8         |

Discussion

Oxalic acid is a virulence factor of several phytopathogenic fungi but the detailed mechanisms by which oxalic acid affects host cells and tissues are not fully understood. OA is widely existing in many biological systems and several studies have shown that the exogenous application of OA mimicked fungal disease prevalence (Qi et al., 2017). The secretion of OA was essential to affect pathogenicity in plants by Sclerotium spp. fungal pathogen infection (Williams et al., 2011) and it has been recorded as an effective elicitor and improving plant resistance (Dong et al., 2008; Monazzah et al., 2018; Sun et al., 2019).

Previous results demonstrated the accumulation of OA can acidify the infected plant tissues to activate many fungal enzymes and protein kinase of host plant cells at low pH and degrade the plant cell wall via acidity or chelation of the cell wall Ca\(^{2+}\) (Grabski et al., 1994; Boller, 1995; Kumar et al., 2021) and the application of exogenous OA modulated the distribution of Ca\(^{2+}\) (Sadak & Orabi, 2015; Li et al., 2016). This was evident in the type of chromosomal abnormalities in our results, OA depletion of Ca\(^{2+}\) leads to spindle fibers malfunction abnormalities such as star metaphase, lagged chromosomes, chromosomal bridge, polyploidy, sticky, and disturbed chromosomes. These abnormalities were increased by increasing the concentration of OA and the time of treatments. The rates of cell division were decrease slightly at low concentrations within the least time duration, but with increasing either the applied concentration or time interval, the reduction was greatly increased. Also, it has been observed that the dynamic changes in Ca\(^{2+}\) spatial and temporary distribution might correlate closely with its distinct roles played during programmed cell death of plants or other plant physiology processes (Borrelli et al., 2016; Gębura & Winiarczyk, 2016).

Fig. 1: Different chromosomal aberrations in root tips of Allium cepa (1-20) induced by 1, 2, and 4 g/L of oxalic acid, and Sclerotium cepivorum crude extract.
The OA appears to function during plant-microbe interaction by triggering the pathways responsible for programmed cell death in plants (Ravi et al., 2017; Fagundes-Nacarath et al., 2018). OA induces cytoplasmic acidification which triggers the synthesis of phytoalexins and other secondary metabolites (Westphal et al., 2019). The cytoplasmic acidification caused DNA breakdown which is evident in the type of chromosomal break, ring chromosome, lagged chromosomes and micronuclei in our results.

Intracellular acidification, combined with $K^+$ and $Ca^{2+}$ flux, was regarded as an early marker of an elicitation process leading to PCD. Several studies suggested that changes in cytoplasmic pH resulting from ion fluxes and $H^+$-ATPase play a role (Afzal et al., 2020). This may explain the severe reduction in mitotic index at all time durations treatments of higher OA concentrations and Sclerotium extract, and the appearance of deformed nuclei and dead cells. These results might suggest that the addition of OA played inhibition role on the inward $K^+$ current and the accumulated $H^+$ in the cytoplasm thus altered the activity of the $K^+/H^+$ exchanger. The Alteration in $H^+$ in the early response of plant cells to environmental stimuli, such as turgor, gravity, pathogen attack and chemicals exposure, have been well explored (Gonugunta et al., 2009).

![Fig. 2: Index rates of mitotic phases in root tip cells of Allium cepa after 6, 12, 18, and 24 hrs exposure to 1, 2, and 4 g/L of oxalic acid, and Sclerotium cepivorum crude extract.](image)

Many physiological events of plant cells, such as nutrient transport across the plasma membrane, cell elongation, and organ development, are highly dependent on the ability of individual cells to control pH both in cytosol and apoplast (Staal et al., 2011). Generally, the modulation of intracellular pH or extracellular pH could lead to depolarization or hyperpolarization in the plasma membrane (Wang et al., 2018). They were subsequently followed by triggering or inhibiting a series of physiological events at the plasma membrane, such as control of ion channels activities, signaling and nutrient uptake, and cell growth (Zhang et al., 2005). In our results, the transduction of such signals leading to the death of onion cells in response to OA treatment which was evident by the decrease of the normal cells and the increase cell mortality at higher OA concentrations. This death displayed characteristic hallmarks of PCD, such as cell shrinkage, deformed nuclei, cleavage of nuclear DNA, and activation of anion channel-dependent, and gene expression (Errakhi et al., 2008). In this study, we confirmed that high concentrations of OA induced PCD in onion cells. The effect of oxalic acid mimics S. cepivorum crude extract on the cytology of Allium cepa germinating roots and the results showed that Mitotic indices were reduced, and the reductions were proportional with both concentration and time. Chromosomal abnormalities related to spindle fiber and protein destruction, Ca ion depletion, DNA break, pH reduction was recorded such as vacuolated nuclei, rod-shape cell, binucleated cells, disrupted nuclei lagged chromosomes, bridge, chromosome polyplody, mega cell, star metaphase, minichromosomes, ring chromosomes, and deformed nuclei during mitosis.

 References


