Production of salt tolerant embryos and cytological changes associated with tolerance in microspores of oilseed rape

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Experiments were carried out to investigate the potential for selecting microspores tolerant to NaCl and subsequently to regenerate pollen-derived plantlets. The oil rapeseed plant *Brassica napus* L.Topas was used in this investigation. Microspores at appropriate stage of development were isolated from anthers and cultured in liquid medium using a range of NaCl concentrations to establish the dose response. The low levels of sodium chloride in culture medium imposed stress on the cultured microspores resulting in symmetrical embryogenic division. Microspore embryogenesis was entirely halted when the culture medium was supplemented with high levels of salt. Ultrastructural survey indicated that microspores grown under saline condition were characterized by accumulation of large number of ribosomes and endoplasmic reticulum in the cytoplasm, formation of thick wall and persistence of large vacuole and presence of numerous lipid droplets. These results indicate that alteration in cytological pattern of cell grown under saline condition is a key factor for regulation of the system that in turn confers resistance to saline stress. The mechanism by which sodium chloride possibly exerts its effect on genes is discussed.

Key Words: Microspore culture, pollen embryogenesis, salt stress, ultrastructure.

Introduction

Application of tissue culture techniques that involve plant cell, tissue and organ cultures, for screening and developing tolerant genotypes have been broadly used in recent years. Cell culture represents unique and useful method to develop stress tolerant cell lines in relatively short time compared to conventional crop improvement programs. Therefore, considerable research has been focused on assessing tolerance in plant using cellular technology. Various types of stress have been studied including salt resistance (Kim *et al*., 1995), disease resistance (Ingram, 1983) and cold tolerance (Chen *et al*., 1982). Salt tolerant plants were produced in Bamboo and Banana (Huang & Huang, 1993; Garcia-Reina *et al*., 1988). Majority of selection studies that carried out *in vitro* focused on using plant materials that comprised somatic cells such as organs, tissues and isolated cells.

Little attention has been paid to the utilization of male reproductive cells in abiotic and biotic experiments. A possible alternative to the conventional method in crop

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improvement is to utilize the reproductive cells that possess only one set of chromosomes. Utilization of the *Brassica* microspore system in selection programs represents a unique and powerful alternative to other *in vitro* selection techniques. Swanson (1989), reported some of the advantages of using microspore culture technique in selection experiments. These advantages include the use of large single cell haploid population, low levels of somaclonal variation, efficient and uniform mutagenic applications, expression of recessive traits and a final product which breeds true for the selected trait. The most valuable characteristic of this technique is that large number of the produced embryos can give rise to plants.

This study focuses on utilization of the isolated microspores of *Brassica napus* L. for induction of salt tolerant embryos and plantlets *in vitro*. Moreover, fine-structural features of the tolerant microspore at different developmental stages and the formed embryos are investigated and compared with non-tolerant counterparts.

Materials and Methods

Plant material

Seeds of *Brassica napus* L. were sown on soil surface in plastic germination trays. Following germination to the four-leaf stage, seedlings were transplanted into 5 inches and then finally 10 inches plastic pots containing a mixture of loam, peat and sand. The plants were grown in the glasshouse, watered and given nutrient feeding solution regularly to maintain optimal growth. Buds at appropriate developmental stage for embryogenesis were collected and microspores were isolated using the method described by Zaki (1999). The isolated microspores were cultured in nutrient liquid medium (Lichter, 1985). The culture medium was supplemented with sodium chloride at the following levels 0, 100, 200, 300, 350, 400 and 450 mM. The cultures were sealed with parafilm and incubated at 30°C for 2 weeks in darkness. Following determination of lethal dose of sodium chloride on cultured microspores, selection experiments were performed using concentration higher than the lethal dose. Mature embryos, tolerant and non-tolerant, were transferred to regeneration medium for plantlet formation. The cultures were maintained at 25±1°C with a 16 h illumination regime (3000 lux).

Electron microscopy

Samples of cultured microspores as well as salt-sensitive and salt-tolerant multi-cellular embryos developed in liquid medium were processed for electron microscopy studies. The samples were fixed for four hours in 2% glutaraldehyde in 0.05M phosphate buffer (pH 7.0) at room temperature. Following fixation, embryos were washed in several changes of buffer, post-fixed in 1% osmium tetroxide for 4 hours at room temperature and dehydrated in graded ethanol/water series. Following passage through a graded propylene oxide/ethanol series, the segments were gradually infiltrated with resin by placing them for 24 hours in each of series of resin/propylene oxide mixtures, followed by three changes in 100% Epon substitute. Finally, materials were embedded in freshly made resin mixture, and polymerized in oven at 60°C for 48 hours. Sections were cut, 0.1µm, mounted on copper grids and stained with uranyl acetate and lead citrate as

described by Reynolds (1963). Observations were made with a Goel Electron Microscope operating at an accelerating voltage of 75kV.

Results and Discussion

Response of the cultured microspores

Earlier studies on microspore culture indicated late uninucleate microspores of *Brassica napus* L.as the optimal stage for culture *in vitro* (Zaki, 1999; Zaki and Dickinson, 1995). Therefore, microspores at this stage of development were isolated and cultured in liquid medium for induction and selection of salt resistant embryos. Within 24 hours in culture, the microspores showed marked heterogeneity where large number of them degenerated and others acquired various features. The latter included enlarged cells, necrotic cells, plasmolysed cells, cytolysed cells and cells that possessed electron opaque cytoplasm. Amongst this population, a small fraction of the cultured microspores continued dividing to form embryos. Therefore, elimination of non-healthy cells that destined for degeneration was essential during this course of study in order to explore cytological characteristics of resistant and non-resistant embryonic microspores.

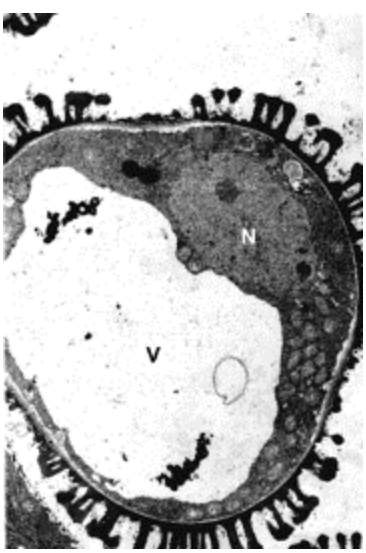
During early stage in culture, the microspores considered to be embryogenic, followed a characteristic pattern of development. The embryogenic microspores remained enveloped by the prominent exine and increased in size. The majority of these cells underwent first division within 2-3 days in culture. The first embryogenic division resulted in formation of two identical cells enveloped by the pollen exine. Interestingly, low levels of sodium chloride, 100 mM and 200 mM, in culture medium was found to provoke microspores to divide symmetrically and switch into embryogenic pathway of development. Moreover, this enhancement in microspore embryogenesis was correlated with increasing embryos formation. On the contrary, microspore embryogenesis was entirely halted when culture medium was supplemented with high levels of salt (300mM). Therefore, this level of sodium chloride in the culture medium was considered as the lethal dose. The response of cultured microspores was also inhibited above the inhibitory level of sodium chloride. This is compatible with the finding of Kirti *et al*. (1991) on *Brassica juncea*, and Nickle and Yeung (1993) on *Daucus carota*, where high levels of sodium chloride halted cellular development.

Selection experiments were carried out by culture of microspores in a medium enriched with 350 mM sodium chloride. Morphologically, there was no difference between symmetrically dividing microspores whether present in medium supplemented or devoid of sodium chloride. Following the first division, the two-formed cells underwent a phase of rapid division forming multicellular embryos. The latter were visible in the culture medium during the second week. A few embryos were apparent in the selection medium supplemented with sodium chloride at 350mM. These embryos were considered salt resistant structures. The formed embryos continued to develop passing through globular shaped embryo and cotyledonary embryo. During this course of study, mature embryos were formed within four weeks in culture medium. These embryos were transferred to regeneration medium for plantlet formation. From earlier studies it emerged that not only is embryogenesis species-dependent, but also heavily reliant on imposing stress on the cultured microspores. Stress treatments have long been employed for induction of pollen embryogenesis in various plant species. Studies as long ago as

1937, Sax, (1937) indicated that stress can lead to symmetrical division, which is a preliminary requisite for pollen embryogenesis in *Brassica napus* L. (Zaki and Dickinson, 1995). In most cases these involve some form of physiological stress or shock, commonly elevated temperatures and high sugar levels (Dunwell *et al*., 1985; Keller *et al*., 1975). Indeed, the low levels of sodium chloride in the culture medium impose stress on the cultured microspores resulting in high number of symmetrical embryogenic division. These results on sodium chloride and its promotive effect on embryogenesis are in agreement with the work of Sasikala *et al*. (1993) who reported on beneficial effect of salt on growth of potato *in vitro*. The work with sodium chloride suggests that it is sufficient, at low concentration, to induce the cultured microspores to divide symmetrically for gametophytic development to be switched off and the sporophytic program to be activated. However, the mechanism by which sodium chloride exerts its effect on genes remains to be explored.

Ultra structural survey

Isolated microspores, at the time of culture, featured a large vacuole often occupying over 50% of the cell volume and the nucleus was pressed to the microspore intine. The cytoplasm of the microspores possessed a complete set of sub-cellular organelles including plastids, mitochondria, dictyosomes, endoplasmic reticulum and ribosomes. Following 12 hours in culture medium, microspores cultured in medium that lacked or supplemented with sodium chloride showed common features. The latter included the presence of large vacuole containing electron-opaque materials and nucleus possessing prominent nucleolus (Figs. 1 & 2). While microspores cultured on medium devoid of salt exhibited translucent cytoplasm (Fig. 1), those cultured in medium enriched with sodium chloride displayed dense cytoplasm (Fig. 2). High magnification view of a portion of the cytoplasm in both cells indicated them to possess smooth and rough endoplasmic reticulum (Figs. 3 & 4). The cytoplasm of cells grown under saline condition was furnished with a large population of ribosomes (Fig. 4). These observations suggest that cultured microspores in saline stress become adapted for unfavorable conditions as early as 12 hours in culture. Earlier studies indicate that protein synthesis in particular proline occurs as a response of culture in saline conditions (Tawab *et al*., 2000). Evidence from the electron-micrographs indicate the rapid and extensive accumulation of ribosomes and rough endoplasmic reticulum in cytoplasm of cells exposed to salt stress. These organelles are known as tools for protein synthesis. The remarkable accumulation of these elements refers to active phase of protein synthesis that in turn reflects activation of genes. This observation may be considered to confirm that acquisition of resistance is related to activation of genes. Following the processes of transcription and translation, a protein is formed in the cell that equip cell to become potentially resistant to the unfavorable conditions. Studies during the last decade indicate that proline is formed in cells exposed to salt stress (Gzik, 1996; Potluri and Prasad, 1993).



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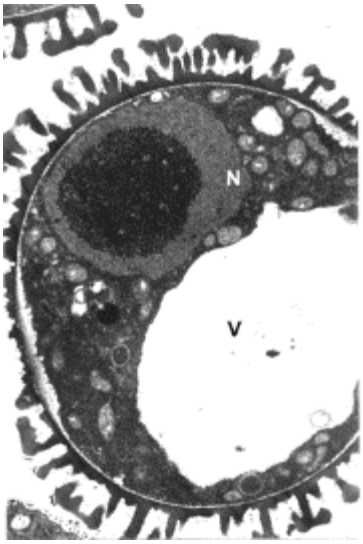

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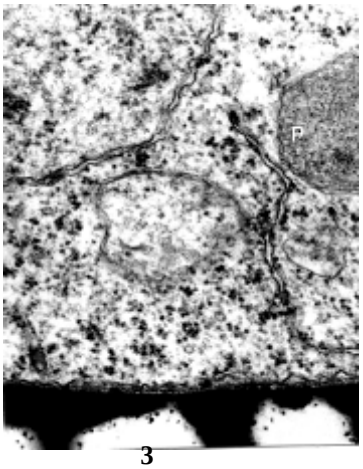

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Fig. (1). Electron-micrograph of a uninucleate microspore prior to first mitotic division. The cytoplasm possesses prominent nucleus (N) and large vacuole (V). (x. 2.500).

Fig. (2). Electron micrograph of a uninucleate microspore, cultured in medium containing high level of sodium chloride, prior to first mitotic division. The cytoplasm possesses prominent nucleus (N) with nucleolus and large vacuole (V). Note the dense cytoplasm of the cell due to accumulation of ribosomes. (x. 3.000).

Fig. (3). High magnification view of a portion of a uninucleate microspore showing endoplasmic reticulum, plastid (P), mitochondria (M) and numerous ribosomes. (x. 11.000).

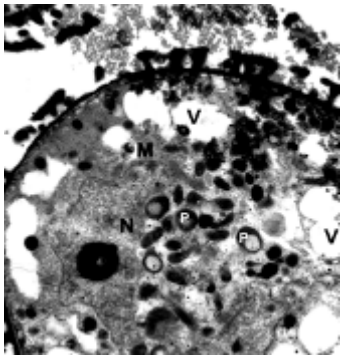
Fig. (4). High magnification view of a portion of a uninucleate microspore, cultured in medium containing sodium chloride, showing rough endoplasmic reticulum, lipid droplets (L) and polysomes. (x. 14.000).

Prior to the first division *in vitro*, the large vacuole was fragmented into numerous small organelles and the nucleus migrated to a central position in the cytoplasm of the cell (Fig. 5). When nucleus acquired central position, first division resulted in formation of two identical cells (Fig. 7). The two-formed daughter cells were enclosed within the exine and each containing numerous small vacuoles, mitochondria, plastids and

nucleus with prominent nucleolus (Fig. 7). On the other hand, uninucleate microspores cultured in medium supplemented with sodium chloride (300mM) featured thick prominent wall and large vacuole that expanded reaching maximum size prior to division. Interestingly, division took place while nucleus was placed at peripheral position and microspore possessed expanded vacuole (Fig. 6). This cellular division gave rise two daughter cells enveloped by prominent thick wall (Fig. 7). Unlike the fragmented vacuole in microspores that cultured in medium free of salt, the large vacuole remained prominent feature for each daughter cell (Fig. 8). The cells possessed large vacuole contained electron-opaque materials, plastids devoid of starch grains and numerous mitochondria with well-developed cristae (Fig. 8).

One of the striking observations in the present work, is the presence of large vacuole and its persistence in salt resistance cells during early stages of development. Similar observation was reported in cantaloupe cells exposed to saline stress (Zaki, 1996). It is obvious that the presence of large vacuole compensates for high osmotic pressure of the culture medium which was enriched with high level of sodium chloride. This modification in vacuolar system is essential for cells to avoid damage and cytoplasmic plasmolysis. Zaki (1996) indicated that vacuole may play an active role in elimination of molecules and ions from cytoplasm that are not required by cell and possibly have lethal effect on the cell. If presence of these elements causes physiological damage to the cells, the elimination of these molecules and ions and their degradation in the vacuolar system would insure survival of the cells at high level of sodium chloride.

The cytoplasm of the bicellular embryo possessed lipid droplets (Fig. 8). Fibrillar wall and middle lamellae separated the two neighbor cells. The first embryogenic division was followed by a phase of rapid activity and cellular division. Proliferation of the two daughter cells formed a multicellular embryo (Figs. 9 & 10). The cells of this structure were very similar to those of the bicellular embryo. Each contained nucleus and subcellular organelles including plastids, mitochondria and vacuoles (Figs. 11 & 12). The multicellular embryo remained enclosed within the microspore exine. Remarkably, plastids of the cells forming embryo, grown on medium devoid of salt, showed extensive increase in starch accumulation (Figs. 9 & 11). On the contrary, starch grains were rare in plastids of salt resistant cells (Figs. 10 & 12). Moreover, the cytoplasm possessed numerous lipid droplets that were absent from the cytoplasm of non-resistant cells. The results present here on formation of thick fibrillar wall are similar to observations of Piqueras *et al.* (1994) and Bressan (1990) who reported deposition of thick cell wall in cells under saline stress. The thick wall may have a vital and unique physical and physiological function that permits cells to survive the external stress in the culture medium. This modification of the cell wall under saline condition indicates the active role of the wall in regulating and channeling the influx of molecules and ions into the cytoplasm. This regulation is a key factor for maintaining integrity of the system and confers resistance to the cells.



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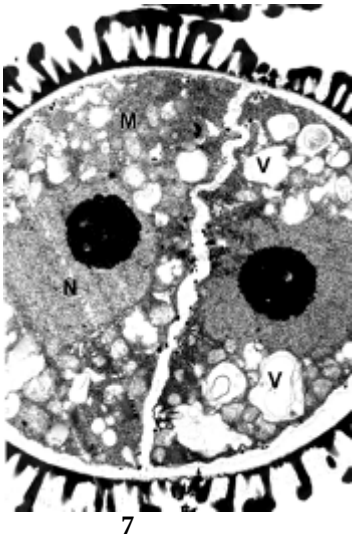

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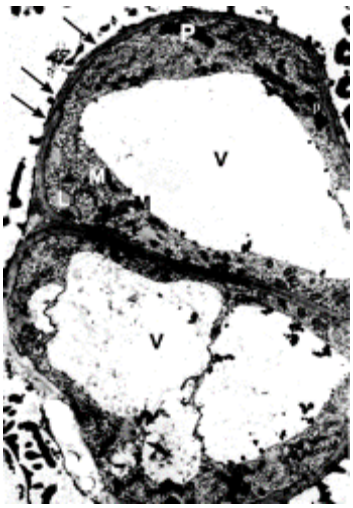

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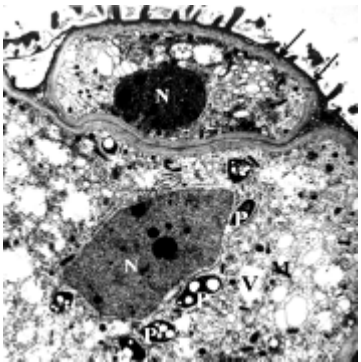
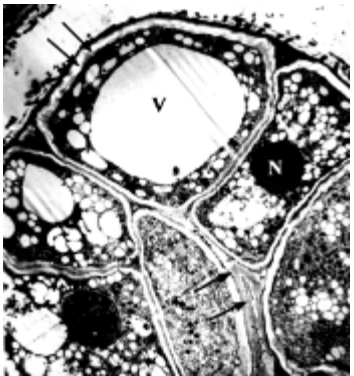
Fig. (5). Electron micrograph of microspore prior to the first mitotic division in medium devoid of sodium chloride. The cytoplasm of the cell contains nucleus (N), mitochondria (M), plastids (P) and numerous small vacuoles (V). (x. 3.000)

Fig. (6). Electron micrograph of microspore, prior to first embryogenic division, cultured in medium containing sodium chloride. The thin layer of the cytoplasm possesses nucleus (N). This layer of the cytoplasm lines the intine and is furnished with numerous ribosomes. Note the formation of a thick wall (arrows) and presence of the large vacuole (v) in the cytoplasm of the cell. (x. 3.000)

Fig. (7). Electron micrograph of bicellular embryo following first embryogenic division in medium free of salt. The two formed cells are identical each possesses large nucleus (N) with prominent nucleolus, numerous small vacuoles (V), and mitochondria (M). (x. 3.000)

Fig. (8). Electron micrograph of bicellular embryo formed in medium containing sodium chloride. The cytoplasm is furnished with numerous ribosomes and contains plastids (P), large vacuole (V), mitochondria (M) and lipid droplets (L). Note deposition of a thick fibrillar wall (arrows) surrounding the two daughter cells. (x.3.500)





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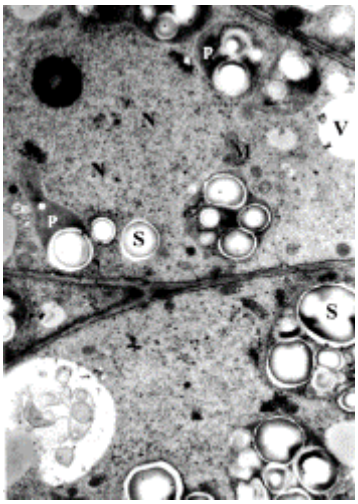

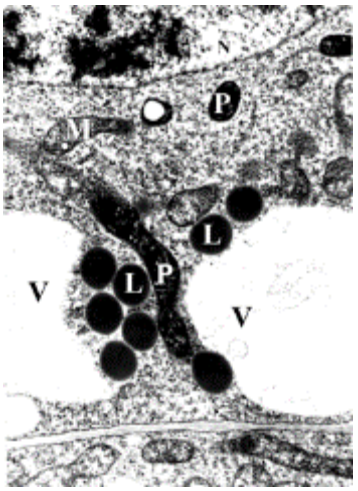

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Fig. (9). Electron-micrograph of a multicellular embryo formed following a number of divisions of a cultured microspore. The proembryo is still enclosed within the pollen exine (arrows). Each cell contains nucleus (N), plastids containing starch grains (P), mitochondria (M) and thick convoluted wall. (x. 4.000)

Fig. (10). Electron-micrograph of a multicellular embryo formed following a number of divisions of a cultured microspore in medium enriched with sodium chloride. The embryo is still enclosed within the pollen exine (arrows). Each cell features nucleus (N), vacuole (V) and dense cytoplasm. Note, the cell wall is deposited investing the cells (arrows). (x. 3.500)

Fig. (11). High magnification at a similar stage to that shown in Fig. 9, revealing the presence of accumulated starch grains (S) in the plastids (P), mitochondria (M) and nucleus (N) with prominent nucleolus. (x. 7.000)

Fig. (12). High magnification view of a portion shown in Fig. 10, revealing the presence of remarkable amount of lipids (L) in the cytoplasm, mitochondria (M), plastids (P) devoid of starch grains and vacuoles (V). (x. 7.000).

One of the other characteristic features of resistant cells is synthesis of lipid droplets and absence of starch grains from plastids. Certainly, this indicates a switch in metabolic pathway of the cells under salt stress. Lipidic materials are considered as a rich

source for energy and provide constant supply of energy for the rapidly dividing embryo. Lipid bodies have also been reported to accumulate in plant tissue growing under salt or water stress (Nazarenko and Serebryakova, 1990; Poljakoff-Mayber, 1981; Valadimirova, 1976). The present results are in harmony with the work of Piqueras *et al*. (1994) who observed lipidic material in the cytoplasm of salt resistant cells in culture. The absence of starch grain from plastids may due to sensitivity of this organelle to salt stress. This is in agreement with the work of Zaki (1996) on cantaloupe and Hayyim and Goffer (1989) on orange tissue. The work indicates that exposure of cells to salt in culture medium interferes with the processes of development in plastids. Indeed, understanding bases for cellular resistance to sodium chloride culture medium would enable utilizing the system for generating salt resistance plants in various plant species.

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