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Attempts of Haploidy Induction in Tomato (*Lycopersicon esculentum* Mill.) Via Gynogenesis II: *In vitro* Non-fertilized Ovary Culture

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Abstract: In order to determine the potentials of *in vitro* gynogenetic induction via *in vitro* ovary culture in tomato a total of 358 ovaries were cultured. Flower buds, of six different cultivars, containing microspores at the uninucleate stage were removed from the plants and following an initial pretreatment on a solid 0.3 M mannitol starvation medium ovaries were cultured on four different media containing ½ MS as the basic medium together with either kinetin riboside or BAP as cytokinins, both in two concentrations, being in combination with the IAA as the auxin also in two concentrations. Ovaries were subcultured on two media, i.e. NLN+2,4-D 1.11 mg l^{-1}+Glycine 500 mgl/l and NLN+NAA 0.93 mg l^{-1} + Casein Hydrolysae 5 g l^{-1}, to induce further sporophytic development. Regeneration neither of somatic nor of gametophytic origin were observed. During culture, ovary walls turned brown and withered but the ovules carried were of two types: one was of globular shape and when observed microscopically showed living cell clumps within the embryo sacs, and the other was of almost rectangular shape comprising shrunk ovules containing dead embryo sac cells within the ovules.

Key words: *In vitro* gynogenesis, ovary culture, mannitol pretreatment, ovule sections

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is the most abundantly produced vegetable crop in the World (Anonymous, 2002). The cultivars used in the production are mainly F1 hybrids because of their high performance from heterosis. Highly homoygote lines are used in the F1 production and 100 % homozygote lines, i.e. pure lines, can be produced via biotechnological methods instead of repeated selfings which generally require many years of time and effort. *In vitro* androgenesis has been the mainstream approach in the production of pure lines via haploids chromosome number of which are later doubled to restore diploidy (Jensen, 1988).

In vitro gynogenesis is an alternative method of haploid induction and can be practiced using non-fertilized ovaries, ovules or the female gametophyte where possible. Interest in this system arises not only from the point of view of haploid induction potentials of the system but also from the maternal origin of the haploids containing cytoplasmic characteristics (Yang and Zhou, 1982, 1990; San and Gelebart, 1986; Mukhambetzhanov, 1997).

Initially, emphasis was given to *in vitro* ovule/ovary cultures by researchers from Maheshwarian school of research between 1958 and 1966 (Yang and Zhou, 1982). Following the opening of the androgenesis era (Guha and Maheshwari, 1964) the other methods of haploid induction were neglected for almost a decade. Following the initial report of Zhu and Wu (1979) on *in vitro* gynogenesis of *Triticum aestivum* successful reports started appearing again on various crops, i.e. *Nicotiana tabaccum* (Wu and Cheng, 1982), *Beta vulgaris* (Hosemans and Bossoutrot, 1983), *Zea mays* (Truong-Andre and Demarly, 1984); *Gerbera jamesonii* (Meynet and Sibi, 1984). Tao *et al*. (1985) produced haploids from non-fertilized ovaries of *Solanum tuberosum*. Alliums (Campion and Alloni, 1990; Keller, 1990 a, b; Bohanec *et al*. 1995) and *Morus alba* (Thomas *et al*., 1999) of the fruit trees were also of interest yielding successful results.

Attempts to produce haploids in tomato have been restricted to in vitro androgenesis and earlier research concentrated only on anther culture but the results obtained were not satisfactory (Chlyah *et al*., 1990). Therefore potentials of non-androgenetic methods of haploid induction in tomato remain to be determined in detail. The only report regarding non-androgenic haploid induction in tomato was that of ovary culture, being cited briefly by San and Gelebart (1986), reporting induction of excess callus from the ovary wall hindering gynogenetic haploid development. Therefore, determination of the potentials of *in vitro* ovary culture may be of use in the production of tomato haploids. In the present work we attempted maternal haploid production via non-fertilized ovary culture in the *Lycopersicon esculentum* Mill.

Materials and Methods

The research was carried out at the greenhouses and tissue culture laboratories of Horticulture Department, Faculty of Agriculture, Cukurova University, Turkey, between February and June, 2000.

General flow of the procedure: Following the removal from the donor plants intact flower buds containing ovaries and microspores at the uninucleate stage were incubated, for the purpose of pre-treatment, on a mannitol starvation medium at 10°C for seven days. After the pre-treatment ovaries were cultured, for 30 days, on media for the induction of switch from gametophytic to sporophytic development of the egg cell. Later ovaries were subcultured to induce further sporophytic development. Data were recorded starting from the initial incubation for pretreatment, and histological observations were carried out on samples obtained at the end of 30 day period of culture on the initial switch medium.

Growing of donor plants: Seeds of open pollinated varieties, i.e. Falcon and Invictus, and F1 cultivars, i.e. Fantastik, Sagit 146, Gokce, and Elif, were sown in seedbeds in early February, 2000. Developing seedlings were planted with 0.4 m and 1.25 m in and between row distances in mid March, 2000. The donor plants were not fertilized during growth.

Culture media and culture practice

Pre-treatment with mannitol starvation medium: As used generally in the androgenetic induction, ovaries were first cultured on a medium containing non-metabolizable carbon source, i.e. mannitol, instead of sucrose. Mannitol of 0.3 M concentration was incorporated into NLN medium (Lichter, 1982) together with ascorbic acid, 50 mg l^{-1}; citric acid, 50 mg l^{-1}; $AgNO_3$, 0.5 mg l^{-1}; Thiamine, 1.0 mg l^{-1}; Nicotinic acid, 5.0 mg l^{-1} and Biotin, 0.1 mg l^{-1}. Agar was used at a concentration of 8 g l^{-1} and pH was adjusted to 5.8 before autoclaving. Heat labile compounds were filter sterilized. Following detachment from the receptacle ovaries were inoculated vertically, the cut surface being in contact with the pre-treatment medium in 9 cm disposable petri dishes and incubated at 10°C in the dark for seven days.

Incubation on initial GI switch media (Stage I): Following pretreatment, in order to induce switch from gametophytic to sporophytic development the GI switch media of the Stage I were prepared using the basic MS (Murashige Skoog, 1962) in half strength, to avoid excess callus formation from somatic tissues. The media contained Indole Acetic Acid and Kinetine Ribozide each being in two concentrations. A total of four media were used, i.e. GI-1: ½ MS + Kinetine Ribozide 1.08 + IAA 0.44 mg l^{-1}; GI-2: ½ MS + Kinetin Ribozide 1.08 + 0.88 mg l^{-1}; GI-3: ½ MS + BAP 2.25 + IAA 0.44 mg l^{-1}; GI-4: ½ MS + BAP 2.25 + 0.88 mg l^{-1}. Other compounds were also added in the media, i.e., the ones added before the autoclaving were $AgNO_3$, 2 mg l^{-1}; sucrose, 30 g l^{-1} and agar 8 g l^{-1} and the others after the autoclaving were l-glutamine, 800 mg l^{-1}; l-serine, l-alanine, l-arginine, l-glycine, all at 100 mg l^{-1}; l-proline, 400 mg l^{-1}, l-asparagine 10 mg l^{-1} and lactalbumine hydrolysate (LH) (Sigma, Edamine K) 10 ml/l. In order to obtain an LH solution 100 g LH was mixed with 300 ml distilled water and the solution was filtered. Ovaries from each of the initial GI media were divided into equal numbers and subcultured on both of the GII media. Samples for histological studies were taken just before the subculture to the GII media.

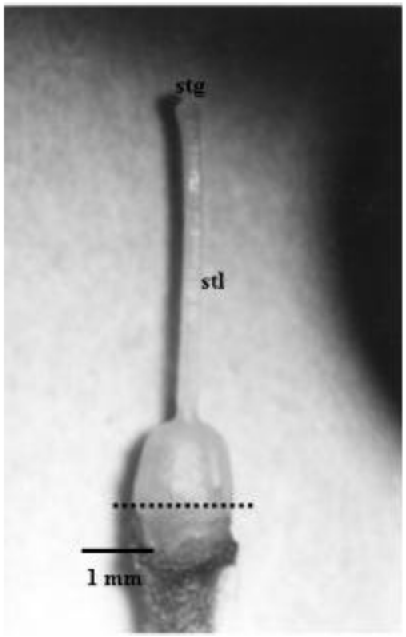

Fig. 1: An ovary just before culture; base was cut from the dotted line and cultured; stg: stigma; stl: style

Subculture media (GII Media) sporophytic development (Stage II): For subculture, basic NLN (Lichter, 1982) medium together with growth regulators and amino acids were used, i.e. GII-1: NLN + 2,4-D 1.11. mg l^{-1} + Glycine, 500 mg l^{-1} and l-Proline 200 mg l^{-1}; GII-2: NLN + NAA 0.93 mg l^{-1} + casein hydrolysate 5 g l^{-1}. Sucrose and agar was added at 30 g l^{-1} and 8 g l^{-1} concentrations, respectively. pH was adjusted to 5.8 before autoclaving. Ovaries were left on the subculture media for about two months until signs of withering were observed.

Stage of flower bud development for culture: Ovaries from flower buds containing microspores at the uninucleate stage were cultured aseptically following removal from the receptacle (Fig. 1).

Histological observations of the embryo sac: Paraffin sectioning was carried out in order to determine any development within the embryo sacs. Ovaries of two cultivars, namely Sagit 146 F1 and Gokce F1, cultured for 30 days on each of the GI media, a total of eight ovaries, were processed according to S tosser *et al.* (1985) and 10 μm sections were stained with haematoxylon dye. Following the staining, sections were observed microscopically.

Data recording and analysis: Ovary diameters, before the pre-treatment, and after culture for 30 days on the GI media were recorded and compared.

Results and Discussion

Following the pre-treatment of starvation, a total of 358 ovaries, from six cultivars, were cultured on the GI media and later were subcultured on the GII media. Regeneration that is neither of somatic nor of gametic origin was determined at the end of the whole culture period. Also, the media employed did not induce any callus development which would possibly overtake the whole ovary hindering any development from the embryo sac, which is contrary to the findings of the only previous report from San and Gelebart (1986).

Mean ovary diameter of varieties varied from 1.6-1.8 mm before the culture to 4.1-4.7 mm after 30 days on the GI media (Table 1). The highest ovary expansion was obtained on the GI-3 medium with an enlargement of 3.0 mm. While the lowest development in the ovary diameter was obtained from the cv. Fantastik to an increase of 3.3 mm on the GI-1 medium, the highest increase in the diameter was on GI-3 medium to 6.3 mm from the cv. Invictus. Despite that the development in the ovary size was initially considered a suitable way of measurement of the performance of the media, it was realized at the experiment's end that enlargement in ovary size may not only be induced by ovule development, which may be expected to push out the ovary wall. It was determined that ovary enlargement provided us with information only on the effects of culture media on the development of somatic ovary wall tissue, rather than from the embryo sacs and this effect is also a genotype dependent as is observed in all somatic tissue cultures. It was therefore thought that ovary development could only be taken into account to measure the performance of the media on somatic tissue development, which was not the purpose of this research.

Table 1: Ovaries and their development on the GI media

Cultivar	Info. On culture	Culture media GI-1	GI-2	GI-3	GI-4
Falcon	A[1]	13	13	12	11
	B	1.7	1.7	1.7	1.7
	C	4.3	4.7	4.7	4.8
Sagit 146	A	24	21	20	21
	B	1.6	1.6	1.6	1.6
	C	4.8	4.5	5.2	4.6
Invictus	A	12	12	13	13
	B	1.8	1.8	1.8	1.8
	C	3.8	3.5	6.3	5.3
Fantastik	A	12	12	11	12
	B	1.7	1.7	1.7	1.7
	C	3.6	3.3	3.7	4.1
Gökçe	A	18	18	18	19
	B	1.8	1.8	1.8	1.8
	C	4.6	5.1	5.0	4.7
Elif	A	13	13	13	14
	B	1.8	1.8	1.8	1.8
	C	3.5	3.5	3.5	3.5
No. OCPM[2]		92	89	87	90
I[3]		1.7	1.7	1.7	1.7
II		4.1	4.1	4.7	4.5
II-I		2.4	2.4	3.0	2.8
Total NOC[4]: 358					

[1]: A: No. of ovaries cultured; B: Average ovary diameter before culture (mm); C: Average ovary diameter before subculture (mm); [2]: No. of ovaries cultured per medium; [3]: I: average ovary diameter on each medium regardless of varieties before culture (mm); II: average ovary diameter on each medium regardless of varieties before subculture (mm); [4]: Total number of ovaries cultured

Table 2: Number and state of ovaries subcultured on GII media

Cultivar	IM / NOC[1]	subcultured on GII-1	subcultured on GII-2
Falcon	GI-1 / 13	5	5
	GI-2 / 13	5	5
	GI-3 / 12	5	5
	GI-4 / 11	4	4
	Σ:49	Σ:19	Σ:19
Sagit 146	GI-1 / 24	12	10
	GI-2 / 21	9	9
	GI-3 / 20	8	8
	GI-4 / 21	9	8
	Σ: 86	Σ: 38	Σ: 35
Invictus	GI-1 / 12	5	5
	GI-2 / 12	5	5
	GI-3 / 13	6	5
	GI-4 / 13	5	6
	Σ: 50	Σ: 21	Σ: 21
Fantastik	GI-1 / 12	4	5
	GI-2 / 12	4	4
	GI-3 / 11	4	5
	GI-4 / 12	4	4
	Σ: 47	Σ: 16	Σ: 18
Gökçe	GI-1 / 18	7	8
	GI-2 / 18	6	7
	GI-3 / 18	8	7
	GI-4 / 19	8	8
	Σ: 73	Σ: 29	Σ: 30
Elif	GI-1 / 13	4	4
	GI-2 / 13	5	4
	GI-3 / 13	4	4
	GI-4 / 14	5	5
	Σ: 53	Σ: 18	Σ: 17
Total		GII-1: 141	GII-2: 140

[1]: Initial medium / number of ovaries cultured

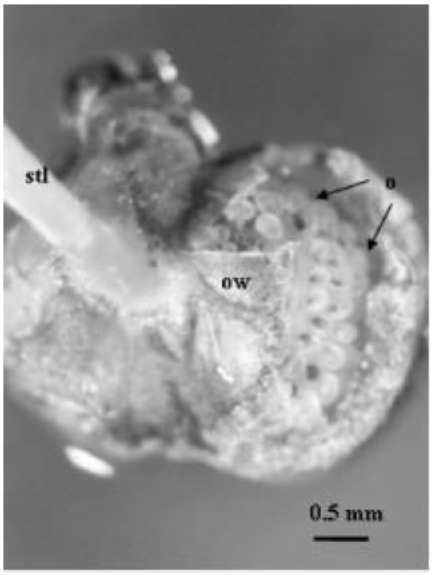

Fig. 2: An ovary on GI medium, ca. 30 days after culture. Ovary wall (ow) broke open exposing the carpel together with globular therefore living ovules (o)

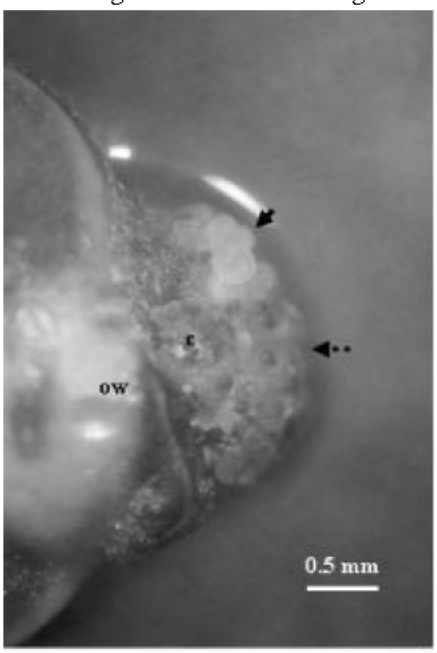

Fig. 3: Ovary wall (ow) receded exposing the whole carpel © out displaying globular and living ovules (arrows) together with shrunk and dead ones (dotted arrow)

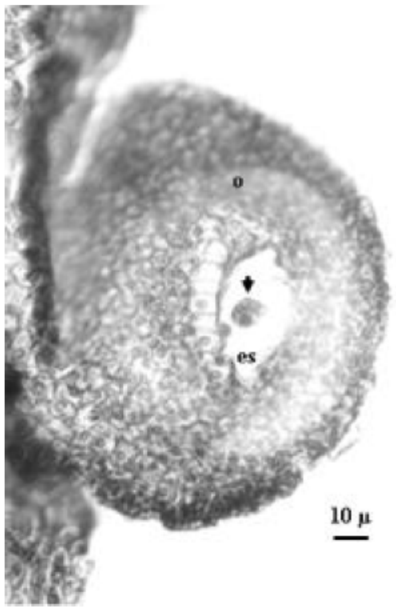

Fig. 4: Transverse section of a globular ovule (o) displaying a cell clump consisting of living cells (arrow) within the embryo sac (es)

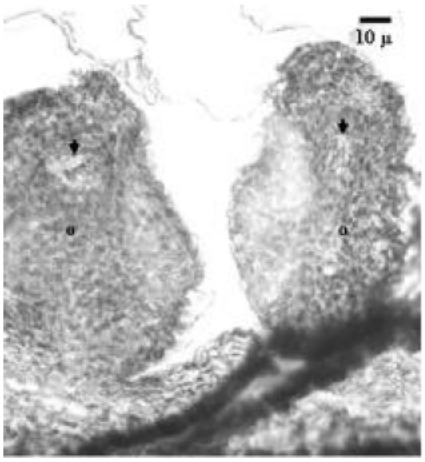

Fig. 5: Transverse section of shrunk and dead ovules (o) displaying collapsed embryo sacs in which only brown and dead cells appear (arrow)

Considering the possible occurrence of haploid induction on the GI media and departing from the view that the gynogenetic induction is a multistage process, the 358 ovaries cultured on each of the GI media were subcultured on both of the GII media that is a total of 141 were on the GII-1 and 140 were on the GII-2 (Table 2). Nevertheless callus development was not observed on any of the ovaries on the subculture media on which they were left for about two months after which most of the ovaries withered and died.

On both of the GI and GII media, together with the ovary wall expansion, ovary walls of some of the ovaries dried and degenerated which restricted ovary wall development and exposed ovules which were then able to develop individually (Fig. 2). The restrictive ability of the media on the ovary walls, if developed and exploited, can help overcome the unfavorable covering and hindering effects

of the ovary walls to the presumably developing ovules underneath. Alternatively, ovary walls can be removed following a certain period of time in culture that is after the ovary walls are expanded outwards enough allowing manipulation. Ovules of such ovaries can then develop without the pressure from the wall. It seems therefore the use of ovules as explant should be practiced whenever possible during culture.

Microscopic observations were carried out on sections of ovules to determine any effects on the cellular level of the cultural conditions. In addition to the morphological appearance of globular and shrunk ovules (Fig. 2 and 3), either circular or rectangular sections of ovules were observed on all the samples studied (Fig. 4 and 5). Coupled with the observation that globular appearing ovules contained living cell clusters within the embryo sacs (Fig. 4) in comparison to the brown dead cells appearing in the shrunk ovules (Fig. 5), it was considered that globular looking ovules were alive at the time of subculture on the GII media and may have remained so for sometime thereafter.

The fact that globular looking ovules remained alive and that somatic tissues did not develop any callus, it was considered that the media and the culture procedure conferred limited success. The procedure applied here maintained the regenerative capacity of the ovules and therefore offers a potential for further manipulations and refinements to the technique.

Effects of mannitol starvation medium at 10 °C on non-fertilized tomato ovaries were not dealt with specifically in our experiments, instead the pre-treatment was directly included in the procedure and therefore it is impossible to reach a conclusion from our experiments. Although a pretreatment approach is not a common practice in the in vitro non-fertilized ovary culture, 8-13°C for rice (Zhou and Yang, 1980; Rongbai *et al.*, 1998) and 10°C for onions (Puddephat *et al.*, 1999) proved useful. Thus a systematic study on the effects of cold and other methods of pretreatment must uncover their potential in the induction of regeneration from the embryo sac.

Yang and Zhou (1990) describes in vitro non-fertilized ovary development as a multi stage process. Instead of a gametophytic one, sporophytic development of embryo sac cells are triggered in the initial stages, and in the followings, ovules/ovaries in which reprogrammed and now sporophytically destined cells, are subcultured to aid further development before a probable abortion. The three kinds of media, subsequently employed in our study, did not result in haploid regeneration from the embryo sac cells. Furthermore, it can be speculated from the microscopical observations of the sections that some cells, exact origin of which is unclear, in the embryo sacs may have been triggered to develop sporophytically. The switch to sporophytic development might have occurred either on the initial pretreatment medium or the GI media. The cell clumps observed in the embryo sacs of ovule sections may be demonstrating multicellular structures leading to proembryo development (Fig. 4). Therefore we can speculate that either the pre treatment or the GI media or both were effective in the induction of the multicellular structures observed, but the GII media were ineffective in the sense that the previously induced structures were not induced further by the subculture media. Therefore the usefulness of the GI media can be suggested with caution only from our results and only further experiments can determine potentials of the media tested here.

As a result, for the first time, in vitro gynogenetic approach to haploid induction in tomato using ovary culture is reported here. Even though limited, success obtained here offers potentials for further refinements in the technique.

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