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Callus induction and plant regeneration of tomato through anther culture

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Abstract

Anthers of 15 genotypes were cultured on MS medium having different concentration of auxins and cytokinins to induce callusing. Of the different media compositions used, callus induction was observed in eight genotypes on MS medium supplemented with 2ip (1mg/l) and IAA (2mg/l). The percent callus formation was maximum in genotype Wild Texas (88.00) and minimum in TLBNRH-1214(4%). The calli obtained from the anthers of the eight genotypes were cultured on different regeneration media. However, the regeneration was observed only in calli of Wild Texas with percent regeneration of 0.66 on MS media supplemented with zeatin (0.5mg/l). After rooting on MS medium containing IBA (0.1mg/l), plantlets were hardened and transferred to pots.

Key words: Growth regulators, organogenesis, *Solanum lycopersicum* L.

Introduction

Tomato is herbaceous, annual, prostate and sexually propagated plant. It is grown in almost every country of the world under open and protected conditions. It belongs to family solanaceae having chromosome number 2n=2x=24. India is the second largest tomato producer of the world after China. The area and production of tomato in India during 2015-16 was about 0.77 million hectare and 18.2 million MT respectively with an average productivity of 20.8 metric tonnes per hectare. Odisha has highest area about 0.070 million hectare, Madhya Pradesh has highest production about 2.17 million MT and Himachal Pradesh has highest productivity about 41.7 metric tonnes per hectare. Tomato is universally treated as 'Protective Food' since it is rich of minerals, vitamins (A, B and C) antioxidants and organic acids (Tigchelaar 1986). It contains approximately 20.50mg of lycopene per 100g of fruit weight (Kalloo 1991). At present, tomatoes are consumed at a higher rate in developed countries than in developing countries and hence it may be referred to as a 'luxury crop'(Sakthivel and Manigandan 2011).Tomato cultivar development may last several years because self-pollinated crops require at least 5–7 generations of selfing (Ellialtiogluet al. 2001). But plant tissue culture techniques are useful instruments in crop improvement. Among these techniques, *in vitro* anther culture is a powerful tool when integrated into breeding programme (Hu and Zeng 1984). Anther culture provides the homozygous double haploid plants within a comparatively short time (Nurhidayah et al. 1996). It can be a powerful shortcut leading to the production of homozygous doubled haploid pure lines in just one *in vitro* generation. It saves time and costs that make this method adaptable in those species where efficient androgenic protocols are well established. Haploids become 100% homozygous after doubling of the chromosome number and can be used as parental lines directly in the production of F_1 lines. Experimental induction of androgenesis was first attempted in tomato (Sharp et al. 1971; Gresshoff and Doy 1972). Sharp et al. (1971) were the first to report obtaining callus and roots but no shoots in tomato anther cultures because of increased level of sucrose. In some studies non-haploid plants of high ploidy level are developed such as 2n, 4n because the plants are originated from the somatic tissue of the anthers (Ancora et al. 1977). Zagorska et al. (1998) tested the androgenetic ability of 85 tomato cultivars, of which 53 were responsive and produced calli and a smaller number of cultivars developed regenerants. Zamir et al. (1980) compared the androgenic responses of 15 recessive mutants (male sterile) and showed that only $ms10^{35}$ mutant was able to produce callus.

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Materials and Methods

The list of genotypes used in the experiment is given in the table 1.For conducting the experiment, floral buds of different sizes (2mm to 8mm) were collected from Vegetable Research Farm, Punjab Agricultural University, Ludhiana and analysed under microscope for the size and meiotic stage of pollen mother cells as described by Segui-Simarro and Nuez (2007).Generally, the anthers containing the late uninucleate to early binucleate stage were cultured. Collected buds were surface sterilized by washing with tap water and then immersing in 0.1 percent mercuric chloride for three to five minutes followed by three washing with autoclaved distilled water. Sterilized buds were placed at different temperatures to enhance androgenesis. Surface sterilized buds were given a low temperature treatment (placed on moistened filter papers) at 4°C and 7°C for duration of 2, 4, 6, 7, 8, and 9 days.Properly dried buds were dissected with the help of forecep and scalpel blade in autoclaved petri plate to remove the sepals, petals and gynoecium. While dissecting there was no damage to the anthers. Anthers were cultured on basal MS media having different combinations of growth hormones (Table 2) and the experiment was replicated thrice. After culturing, the petri plates were covered with parafilm and the cultures were incubated under complete dark for callus induction. Once the callus got induced, it was subcultured for 2-3 times on same media that was used for callus induction. After 2-3 subculturings the callus was cultured on different regeneration media having different combinations of growth hormones (Table 2). The well-developed shoots were transferred to rooting medium (half strength MS medium containing 0.1mg/l IBA). After sufficient development of shoot and root systems, the small plantlets were taken out from the culture vessels and washed to remove excess of agar around the roots. The plantlets were transferred to plastic cups containing MS basal medium having half strength and were covered with polyethylene to maintain a condition of high humidity (85% RH). Hardening was done at 25 ± 2°C under 16/8 h photoperiod and grown for 2 weeks before planting to the greenhouse. After the development of new leaves, the covers were removed, and hardened plants were transferred to earthen pots filled with soil mix (peat, perlite, and vermiculite in equal proportions: 1:1:1, v/v/v) and grown to maturity.

Results and Discussion

Standardization of size of bud and anther: Previously it has been reported that the microspore development stage from metaphase I to telophase II was the optimal stage for callus induction (Segui-Simarro and Nuez 2005). The bud size was correlated with the microspores at late uninucleate to early binucleate stage of development (Fig. 1 and 2). But there was poor correlation between bud length and stage of microspore development. Earlier reports also differ on size of bud which contains microsporesat an early stage of meiosis (Gresshoff and Doy 1972; Sharp et al. 1972). Therefore, anther length was used as a criterion to select the microspores at appropriate stage of development. Summers et al. (1992) also observed that anther length is the better predictor of callus induction and growth. Anthers of different lengths covering the different stages of microsporogenesis were cultured. However, anther lengths in the range of 2mm to 5mm confirmed the presence of dividing meiocytes in the anther locules and produced callus in the responding genotypes which was similar to as described for haploid production in tomato by Segui-Simarro and Nuez (2007).

Fig. 1: Bud sizes ranged from 2mm to 8mm

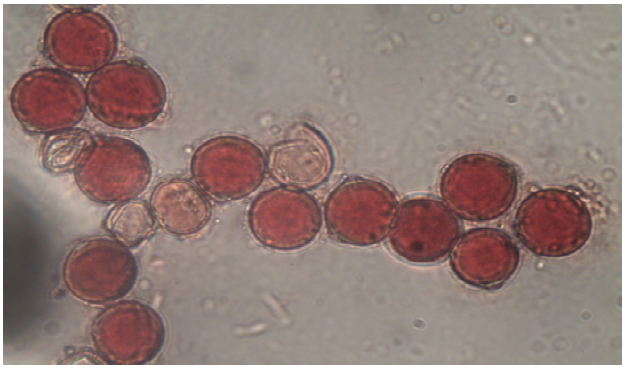


Fig. 2: Late uninucleate to early binucleate stage of microspores

Effect of media composition on callus induction: After 20-25 days of culturing white friable callus masses could be observed to emerge from the locule of swollen anthers (Fig. 3) on callus induction media.It has been observed that a balance between auxin and cytokinin in artificial medium determines the callogenesis. Therefore, MS media supplemented with different concentrations of auxins and cytokinins were used for callus induction. Of the different media compositions used, callus induction in all the responding genotypes was observed only in MS media supplemented with 2ip (1mg/l) and IAA (2mg/l). Earlier also, MS media having different concentrations of combination of 2ip and IAA growth

Wild Texas Roma

Punjab Ratta Punjab Red Cherry

Fig. 3: Callus induction from anthers in different tomato genotypes

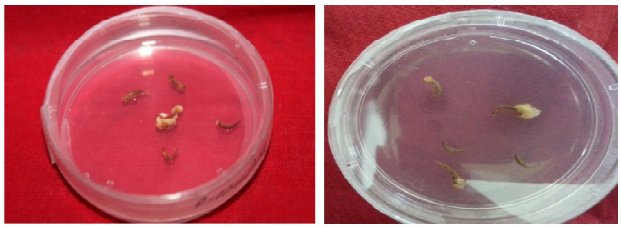
regulators was found favourable for callogenesis in tomato anthers (Shtereva et al. 1998; Segui-Simarro and Nuez 2007). Other media compositions showed no response for callus formation in all the genotypes.In the present study, highest per cent callus formation (88.0) was obtained in genotype Wild Texas (Fig. 7).

Effect of genotype on callus induction: Of the total 31 genotypes assessed for anther culture, callus induction was observed in eight genotypes viz. Wild Texas, Punjab Red Cherry, Punjab Ratta, Roma, Punjab Chhuhara, Punjab Sartaj, TLBNRH-1014 and TLBNRH-1214.Tomato is well known for its recalcitrant nature to androgenesis (Segui-Simarro et al. 2011; Zagorska et al. 2004). In the present study, among the genotypes, the percent callus formation was maximum in Wild Texas (88.00) followed by Punjab Red Cherry (72.00), Punjab Ratta (47.33) and Roma (22.00). Callus induction was minimum (4%) in TLBNRH-1214 (Table 1). Statistically significant differences in per cent callus induction were observed among all the genotypes, except between genotype Punjab Sartaj and Punjab Chhuhara which do not differ significantly from each other. Genotypes TLBNRH-1014 and TLBNRH-1214 were at par for callus induction. Hence, the genotype effect was observed for callus induction. Previously also, it was observed that the responsiveness of the cultured anther to meiocyte callogenesis and organogenesis induction depends upon the donor genotype.

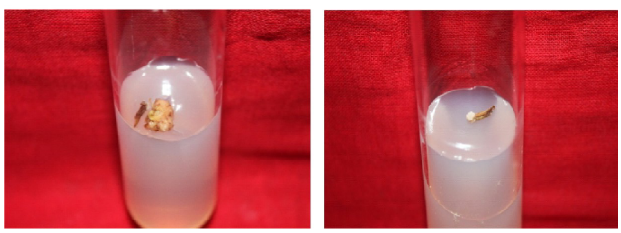
Table 1: The list of genotypes used in the experiment

Cultivated species	Varieties/Lines/Hybrids
Solanum esculentum var *esculentum*	**Varieties:** Roma and Punjab Ratta **Hybrids:** TH-1,TLBNRH-1214, TLBNRH-1014
Solanum esculentum var *cerasiforme*	Punjab Red Cherry, Punjab Kesar Cherry and Punjab Sona Cherry
Solanum esculentum var *pyriforme*	Punjab Chhuhara
Solanum esculentum var *grandifolium*	EC 971784
Solanum esculentum var *validium*	Punjab Gaurav, Punjab Sartaj
Wild species	
Solanum pimpinellifolium	TL 2213
Solanum peruvianum	Wild Texas and EC 717323

Regeneration of shoots from callus: The regeneration was observed only in MS media supplemented with zeatin (0.5mg/l). The cultures were incubated in light for regeneration of shoots from calli. On exposure to light, after 3 weeks of incubation, green regions appeared on the surface of calli from Wild Texas and after 6 weeks shoots were regenerated from these viable calli (Fig. 4). While in genotypes: Punjab Sartaj, Roma and Punjab Ratta the calli on regeneration medium, continued to proliferate into undifferentiated growth without the induction of any shoot bud and finally died. Whereas in genotypes: Punjab Red Cherry, Punjab Chhuhara, TLBNRH-1014, TLBNRH-1214 calli did not grow, became brown and died (Fig. 5). Therefore, of the eight genotypes which responded to callogenesis, regeneration was observed only in calli of Wild Texas with percent regeneration of 0.66. Hence, the genotype has marked

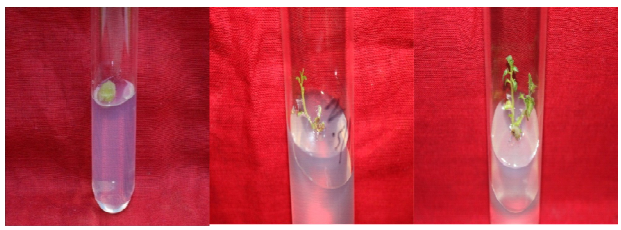


Punjab Chhuhara Punjab Sartaj



TLBNRH-1014 TLBNRH-1214

Fig. 3: Callus induction from anthers in different tomato genotypes



After three weeks After four weeks After six weeks

Fig. 4: Greening of callus and shoot regeneration from anther calli in Wild Texas

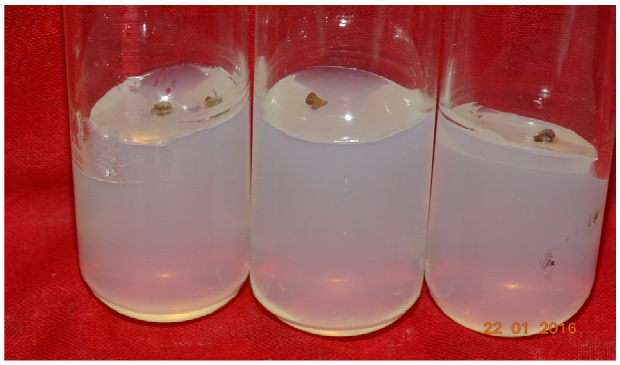


Fig. 5: Browning of anther calli which is not regenerated into plants

effect on plant regeneration from tomato anther calli. Although four studies (Gresshoff and Doy 1972; Ziv et al. 1984; Zagorska et al. 2004; Segui-Simarro and Nuez 2005) have reported plant regeneration from androgenic calli of tomato anthers but in the present study it worked only in one genotype. Thus, it seems clear that tomato is strongly recalcitrant to anther and microspore culture for the production of haploid and doubled haploid plants.

Rooting and Hardening: Segui-Simarro and Nuez 2007 reported spontaneous induction of roots in some genotypes while in others rooting is induced by transferring regenerated shoots in rooting medium. In the present study, roots were not induced spontaneously in the regeneration medium, thus for rooting, the elongated shoots were transferred to half strength of MS basal medium containing IBA (0.1mg/l). The rooted plants were gently removed from the tubes, adhered agar medium was removed by washing with distilled water to avoid contamination. The hardening of the plants was done for two weeks in MS medium having half strength at 25 ± 2°C. The hardened plants were then transferred to plastic cups containing a sterile soil, sand and vermiculite mixture (1:1:1, v/v/v), and after 2 weeks, they were transferred to pots (Fig. 6).

Table 2: List of different culture media used

Type of medium	Composition of media
Callus Induction medium	MS + IAA (2mg/l) + 2ip (1mg/l)
	MS + NAA (2mg/l) + Kin (1mg/l) + 2,4-D(2mg/l)
	MS + NAA (2mg/l) + Kin (1mg/l)
	NLN + BAP (0.5mg/l) + NAA (0.5mg/l)
	MS + NAA (2.5mg/l) + Kin (0.5mg/l)
Regeneration medium	MS +BAP (2.0mg/l) + NAA(0.2mg/l)
	MS + Zeatin (0.5mg/l)
	MS + BAP (1.5mg/l)
	MS + GA_3(1mg/l) + BAP(0.05mg/l) + NAA(0.1mg/l)

Table 3: Response of different genotypes for percent callus induction in tomato

Genotype	Number of anther cultured	Number of calli obtained	Percent callus induction ± S.E
Media: 2ip (1mg/l) + IAA (2mg/l)			
Wild Texas	150	132	88.00 ± 0.94
Punjab Red Cherry	150	108	72.00 ± 1.88
Punjab Ratta	150	71	47.33 ± 1.44
Roma	150	33	22.00 ± 0.94
Punjab Sartaj	150	25	16.66 ± 1.08
Punjab Chhuhara	150	22	14.66 ± 1.44
TLBNRH-1014	150	9	6.00 ± 1.62
TLBNRH-1214	150	6	4.00 ± 0.94
CD at 5%			**4.58**

Fig. 6: Hardening of anther derived plants

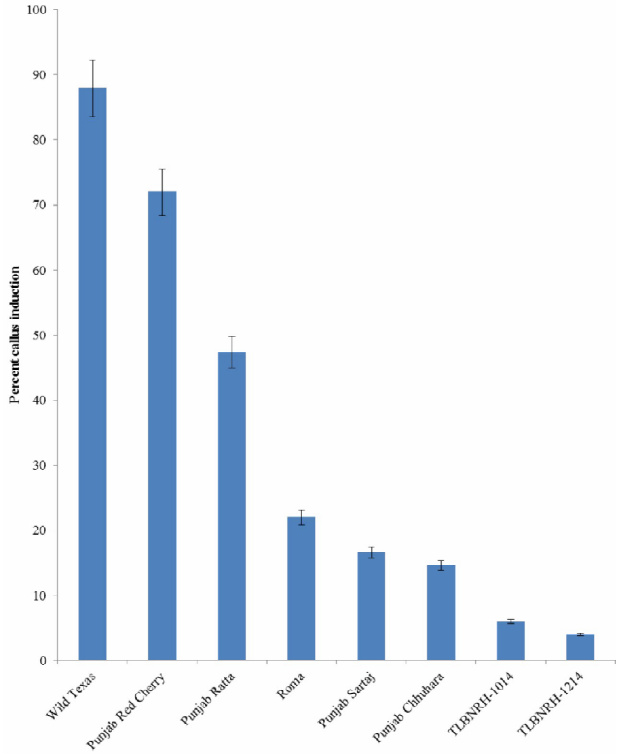


Fig. 7: Effect of different genotypes for callus induction (%) in tomato

Conclusion

This study concluded that bud sizes ranged from 2mm to 5mm having late uninucleate to early binucleate stage were more responsive to callus induction and the

genotype Wild Texas showed the maximum callus induction percentage and also the plant regeneration. Other genotypes were not responded to plant regeneration. The protocol thus developed in the present investigation may significantly contribute to genetic improvement of tomato.

सारांश

टमाटर के 15 प्रभेदों के परागकोशों को एमएम माध्यम पर आक्जिन एवं साइटोकाइनिन्स के विविध सान्द्रता से कैलस बनने हेतु उत्प्रेरित किया गया। परीक्षण किये गये विविध माध्यम संयोजनों में आठ प्रभेदों का एमएम माध्यम जिनमें 2 आईपी (1 मिग्रा./ली.) तथा आईएए (2 मिग्रा./लीटर) के प्रयोग से कैलस उत्प्रेरित हुए। अधिकतम कैलस बनने की प्रवृत्ति प्रभेद वाइल्ड टेक्सास (88.00) तथा सबसे कम टीएलबीएनआरएच–1214 (4.0 प्रतिशत) पाया गया। आठ प्रभेदों के परागकोशों से प्राप्त कैलस का पुनः संजीवित विभिन्न माध्यमों पर उत्पन्न किया गया जबकि पुनः संजीवन कैलस केवल वाइल्ड टेक्सास 0.66 के एमएस माध्यम जिनमें जियाटिन (0.5 मिग्रा./लीटर) था, पर पाया गया। एमएम माध्यम जिनमें आइबीए (0.1 मिग्रा./लीटर), के उपयोग से जड़ विकास के उपरान्त कर्त्तोतकों को कठोरीकृत कर गमले में स्थानान्तरित किया गया।

References

Ancora G, Sree Ramula K and Devreux M (1977) *In vitro* culture of anthers and stems internodes of *L. esculentum*: nuclear DNA determination in calli and cytological analysis of regenerated plants. Zeitschrift für Pflanzenphysiologie 82:377-388.

Ellialtioglu S, Sari N and Abak K (2001) Production of Haploid Plants. In: Babaoglu M, Guzel E, Ozcan S (ed) *Plant biotechnology I*. Selcuk University Press, Turkish. Pp 137-89.

Gresshoff P M and Doy C H (1972) Development and differentiation of haploid *Lycopersicon esculentum* (tomato). Planta 107:161-70.

Hu H and Zeng J Z (1984) Development of new varieties via anther culture. In: Ammiratto P V, Evans D A and Sharp W R (ed). *Handbook of Plant Cell Culture*. Macmillan Publishing Company, New York. Pp 65-90.

Kalloo G (1991) *Genetic Improvement of Tomato*. Springer-Verlag, Berlin, Heidelberg, New York. Pp. 1-9.

Nurhidayah T, Horn R, Rocher T and Friedt W (1996) High regeneration rates in anther culture interspecific sunflower hybrids. Plant Cell Reports16:167-73.

Sakthivel S and Manigandan V (2011) Tissue culture studies in tomato (*Lycopersicon esculentum*) from cotyledonary leaf explants.International Journal of Chemistry and Pharmaceutical Sciences 2(3):22-25.

Seguí-Simarro J M (2010) Androgenesis revisited. Botanical Review76: 377–04.

Seguí-Simarro J M and Nuez F (2005) Meiotic metaphase I to telophase II is the most responsive stage of microspore development for induction of androgenesis in tomato (*Solanum lycopersicum*). Acta Physiologiae Plantarum27:675-85.

Seguí-Simarro J M and Nuez F (2007) Embrogenesis induction, callogenesis and plant regeneration by *in vitro* culture of tomato isolated microspores and whole anthers. Journal of Experimental Botany 58:1119-32.

Seguí-Simarro J M, Martinez P, Vega V and Garci B (2011) Androgenesis in recalcitrant solanaceous crops. Plant Cell Reports 30:765-78.

Sharp W R, Dougall D K and Paddock E F (1971) Haploid plantlets and callus from immature pollen grains of *Nicotiana* and *Lycopersicon*.Bulletin of the Torrey Botanical Club 98:219–22.

Sharp W R, Raskin R S and Sommer H E (1972) The use of nurse culture in the development of haploid clones of tomato. Planta 104:357-61.

Shtereva L A, Zagorska N A, Dimitrov B D, Kruleva M M and Oanh N K (1998) Induced androgenesis in tomato (*Lycopersicon esculentum* Mill.) factors affecting induction of androgenesis. Plant Cell Reports 18:312-17.

Summers W L, Jaramillo J and Bailey T (1992) Microspore development stage and anther length influence the induction of tomato anther callus. HortScience 27:838-40.

Tigchelaar E C (1986) *Tomato breeding*. In: Bassett M J (ed) *Breeding Vegetable Crops*.. AVI publishing Company, Westport, Connecticut. Pp 135-71

Zagorska N A, Shtereva L A, Kruleva M M and Dimitrov B D (1998) Induced androgenesis in tomato (*Lycopersicon esculentum* Mill.) I. Influence of genotype on androgenetic ability. Plant Cell Reports 17:968-73.

Zagorska N A, Shtereva L A, Kruleva M M, Sotirova V G, Baralieva D L and Dimitrov B D (2004) Induced androgenesis in tomato (*Lycopersicon esculentum* Mill.). III. Characterization of the regenerants. Plant Cell Reports 22:449-56.

Zamir (1980) Anther culture of male-sterile tomato (*Lycopersicon esculentum* Mill.) mutants. Plant Science Letters 17:353-61.

Ziv M, Hedary D and Kedar N (1984) *Lycopersicon esculentum*: trifoliate plants recovered from anther culture of heterozygous *tftf* plants. Plant Cell Reports 3:3-10.