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Efficient *in vitro* Callus Induction and Regeneration of Different Tomato Cultivars of India

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Abstract: The present study was conducted to formulate single rapid *in vitro* callus induction and regeneration system for six different commercially important tomato cultivars of India. This study was done as a pre-requisite for the genetic manipulation studies to enrich vitamin E in tomato, which is ongoing research in the group. The varieties in this study include Sindhu and Shalimar (high altitude hybrid varieties), CO_3, PKM, Vaishnavi and Ruchikar are hybrid varieties which are widely cultivated in the different agro climatic regions of India. Effect of the hormones, NAA (Naphthalene Acetic Acid), IAA (Indole Acetic Acid) in combination with BAP (Benzyl Amino Purine) and Kin (Kinetin) at varying concentrations were investigated on callus induction frequency. The varying concentrations of BAP were tested for regeneration capacity in all the cultivars. The highest frequency compact of callus formation was up to 90% for all the cultivars in 0.5 mg L^{-1} NAA+2 mg L^{-1} of BAP and regeneration was significantly higher with 3 mg L^{-1} BAP for all the cultivars. Hence, from this study, we could conclude that even though there are genotypic differences among the cultivars, common media and hormonal concentration can be used for the callus induction and for plant regeneration. In future, these results will be very useful for all the tomato genetic engineering studies.

Key words: NAA, IAA, BAP, callus, plant regeneration, cultivars

INTRODUCTION

Lycopersicon esculentum (tomato) is a major vegetable crop, which is known commonly, only from the last century, used both as fresh and processed food. During the last two decades many biotechnological approaches have been focused on the improvement of tomato crop, which can grow in different agro climatic zones to meet the demands (Mandal and Sheeja, 2003). Plant tissue culture being an important tool in biotechnology and facilitator system for genetic transformation, it is essential to arrive at the common *in vitro* system which can suit most varieties available in the country. The regeneration ability of a number of tomato cultivars have been tested for their ability to produce callus and shoot induction in earlier studies (Costa *et al*., 2000; Venkatachalam *et al*., 2000). Plant regeneration in tomato is via shoot organogenesis from callus (leaf or cotyledon explants) or directly from inflorescence or anther culture under the influence of different phytohormones, but the conditions for *in vitro* plant regeneration is still largely an empirical process and it is difficult

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to achieve for some genotypes within a species (Compton and Veilleux, 1991). The degree of response of explants have been by Durzan (1984) in the order of leaves, cotyledons, hypocotyls, whereas Plastira and Perdikaris (1997) had reported in the order of hypocotyl, cotyledon and leaves. Tissue culture studies with different cultivars of tomato involving different combinations of plant growth regulators have been reported by Bhatia *et al.* (2004) and Dagmara *et al.* (2005).

Although, there are many reports on tomato tissue culture, but only very few studies have been reported in Indian cultivars and in particular their have been no reports especially on high altitude Indian cultivars like Sindhu and Shalimar which are cultivated in Northern Himalayan regions of India (above 8000 m of mean sea level), Vaishnavi and Ruchikar PKM_1 and CO_3 are hybrid varieties which are cultivated in the plains of Southern India. Since, these varieties are widely used, we have developed an optimized culture condition for callus induction, multiple shoot induction and indirect regeneration.

MATERIALS AND METHODS

The seeds of high altitude tomato (*Lycopersicon esculentum* Mill cv.) cultivars viz., Sindhu and Shalimar were procured from Defence Agricultural Research Laboratory (DARL) Pittorgarh, India, the temperate varieties CO_3 and PKM_1 was procured from Tamilnadu Agricultural University (TNAU), Coimbatore and the Vaishnavi and Ruchikar were procured from Directorate of Seed Certification, Coimbatore, India.

Surface sterilization was carried out and seeds were inoculated in half strength Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) with 3% sucrose and solidified with 0.8% agar. The seeds were initially kept in the dark at 27±1°C for two days and then maintained under a 16 h photoperiod at 50 μmol m sec, with day/night temperature of 25±1°C.

Callus Induction

Callus was induced from 10 days old *in vitro* germinated seedlings. Leaves, hypocotyl and stem explants were used. After trimming the peripheral regions, the explants were placed horizontally on the surface of MS medium with different concentration and combinations of plant growth regulators viz., Individual combination of NAA (0.1, 0.2, 0.3, 0.4 and 0.5 mg L^{-1}) with BAP (1, 2, 2.5 and 3 mg L^{-1}) and individual combination of IAA (0.1, 0.2, 0.3, 0.4 and 0.5 mg L^{-1}) with BAP (1, 2, 2.5 and 3 mg L^{-1}) and the combination of NAA and IAA (0.1, 0.2, 0.3, 0.4 and 0.5 mg L^{-1}) separately with different concentrations of kinetin tried (1, 2, 2.5 and 3 mg L^{-1}).

The high altitude cultivars were incubated at 18±2°C while the other cultivars were incubated at 25±2°C with a photoperiod of 16 h at 50 μmol m sec. Selection of hormone and their concentration were based on the earlier reports (Gubi *et al.*, 2004; Cappadocia and Ramulu, 1980; Bhatia and Ashwath, 2004; Jose *et al.*, 2007). A control group was also maintained (MS basal medium without plant growth regulators) to record the callusing frequency response.

Plant Regeneration from Callus

The 25 days old calli (derived from leaves, hypocotyl and stem) were transferred to regeneration medium containing MS with various hormones at different concentrations singly, viz., 1, 2, 3, 4 BAP mg L^{-1}. The high altitude cultivars were incubated at 20±2°C while the other cultivars were incubated at 25±2°C with a photoperiod of 16 h at 50 μmol m sec.

Statistical Analysis

Experiments were set up in a randomized block design and each experiment had 5 replicates and repeated for 3 times. Six explants were used per treatment in each replicate. Observation was recorded on the frequency of callus induction and regeneration (in days). The analysis was done by taking mean and standard deviation followed by t-test (paired two samples for means). The design was carried out to detect the significance of differences (5% level of significance) among the treatment means.

RESULTS AND DISCUSSION

Seed Germination and Callus Induction

The seeds of all cv. started to germinate in half strength MS media, after a week the young seedling grew with emergence of cotyledons, green elongated hypocotyls and roots.

Among the tested NAA with BAP combinations for callus induction for different cultivars using different explants (leaves, stem and hypocotyl), 0.5 mg L^{-1} NAA+2 mg L^{-1} BAP showed rapid and maximum *in vitro* response.

A significant increment of callus proliferation was observed over the culture period. The frequency and percentage (upto 90%) of callus induction was observed maximum in hypocotyl than leaves and stem (Fig. 1a-c) with aforesaid combination of growth regulators. Difference in the initiation of callus formation such as curling of leaf, bulging of stems and formation of mass of cell aggregates at the peripheral regions were observed within 6-9th day for all the cultivars. Well developed callus were observed between 18th-22nd day for the all the cultivars (Fig. 2a-d). The calli were subcultured at 30 days interval in same medium. In terms of morphology of the callus, it was green and hard in medium supplemented with 0.5 mg L^{-1} NAA and 2 mg L^{-1} BAP and soft, fragile and greenish yellow in the 0.5 mg L^{-1} IAA and 2.5 mg L^{-1} BAP treatment.

Plant Regeneration

The medium supplemented with 3 mg L^{-1} BAP was the best for induction of shoots which occurred within 2nd week from hypocotyl explants in all the cultivars compared to leaf and stem explants. The percentage response of shoot formation was maximum in the order of hypocotyl>leaf>stem in 3 mg L^{-1} BAP than other combinations for all the cultivars. However; there were significant differences in regeneration capacity between the cultivars in terms of frequency, number and size of shoots regenerated, as well as in the duration required for regeneration (Fig. 3a-c). A total of 5 to 10 shoots with 2 to 4 cm in height were observed after 4th week for all the cultivars (Fig. 4a-f).

The result of this study demonstrates the callus induction and shoot regeneration for all 6 commercial by valuable tomato cultivars with a single media and hormone combination. In previous reports genotypic variation was observed among the tomato cultivars in terms of callus and regeneration responses (Bhatia *et al*., 2005).

All the earlier reports shows that the good and faster response of callus are formed only in medium having the equimolar concentration of auxin and cytokinins (Chaudhry *et al*., 2004), which are contrary to our present findings. Present findings show that the callus is induced in high concentration of cytokinins compared to concentration auxin (Hill *et al*., 1989). The auxin/cytokinin (1/4) proportion, used in the present study (0.5 mg L^{-1} NAA+ 2 mg L^{-1} BAP) increased callus induction significantly and in higher frequency. The same media is also found to be capable of maintaining the long term callus growth and development unlike others was reported by Rogozinska and Skutnik (1974) and Imanishi *et al*. (1976).

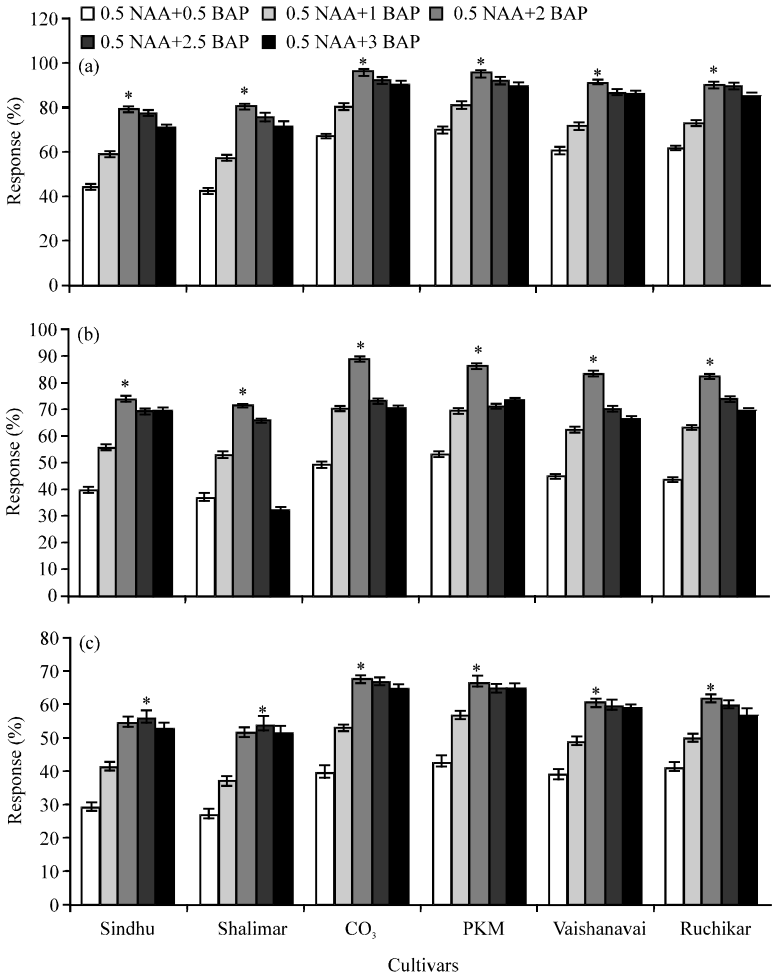

Fig. 1: (a-c) Percentage of response for callus induction under the influence of different concentration of hormones. 0.5 NAA mg L^{-1}+2 BAP mg L^{-1} shows the maximum response in the all explants, {(a) hypocotyl, (b) leaf and (c) stem} of the six cultivars

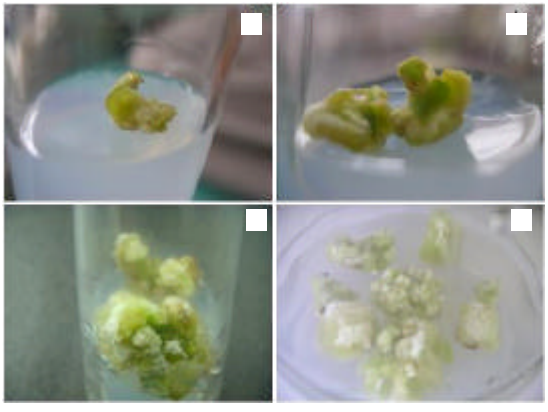

Fig. 2: (a, b) Different stages of callus formation and (c, d) callus culture

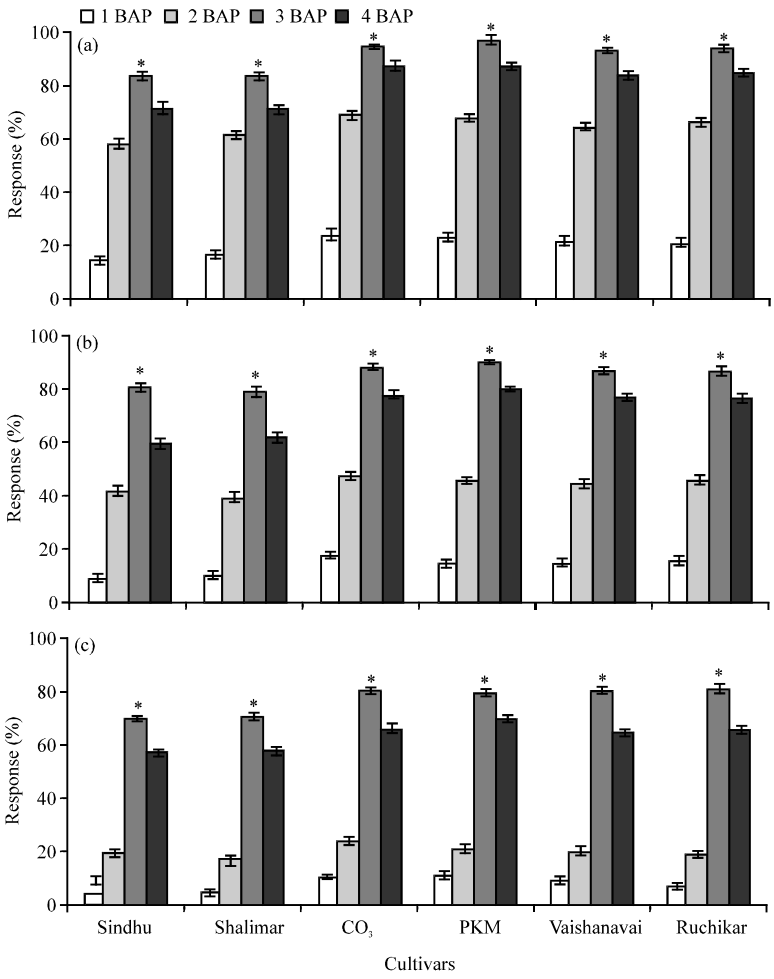

Fig. 3: (a-c) Percentage of response for regeneration under the influence of different concentration of hormones in 4 weeks. 3 BAP mg L^{-1} shows the maximum response in the all explants {(a) hypocotyl, (b) leaf and (c) stem} of the six cultivars

Fig. 4: (a-f) Different stages of plant regeneration from the callus culture

In Solanaceae members, the auxin alone or in combination with a cytokinin, shown to induce the formation of calli. The importance of the hormonal balance in tomato has been shown critical in several reports, as in the case of present results (Canhoto *et al*., 1990).

The medium containing auxin with kinetin was reported as the most efficient for callus formation in tomato (Gulshan *et al*., 1981). In this study, we investigated the influence of NAA and IAA with kinetin for callus induction for all the six cultivars. In contrast, our results showed no considerable response on callus induction by the auxin and kinetin combinations.

Among the various explants used, hypocotyl was best in terms of average number of shootlets, which could probably be attributed to the age compared to other explant leaf and stem. Younger explants showed better callus induction and organogenetic response (George and Sherrington, 1984). The importance of hypocotyl derived callus in terms of plantlet regeneration corroborates with the earlier findings of Locy (1981). A similar type of comparison for genotype and explant type selection and 70 to 80% regeneration from hypocotyls were reported by Gubis *et al*. (2003), Moghaleb *et al*. (1999) and Jabeen *et al*. (2005). As many reports suggest the use of zeatin as primordial phytohormone for regeneration alone or in combination with auxin, we suggest the BAP as an cheaper alternate.

Optimization of callus induction and plant regeneration for commercial cultivars and breeding lines will significantly aid in the development of a genetic transformation system, which is the primary aim of this study.

In conclusion, present study suggests that even though there are genotypic differences among the cultivars, single hormonal concentration can be used for the callus induction and plant regeneration, which will aid in genetic transformation studies.

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