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Improving the Quality of *in vitro* Cultured Shoots of Tomato (*Lycopersicon esculentum* Mill. cv. Red Coat)

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Abstract: Effects of activated charcoal (0, 1, 5 and 10 mg L^{-1}), ascorbic acid (30, 60, 120, 240 and 480 μM) and casein hydrolysate (0, 50, 100, 200, 500 and 1000 mg L^{-1}) were evaluated for morphogenesis of *Lycopersicon esculentum* cv. Red Coat cotyledons. The media containing higher concentrations of activated charcoal and ascorbic acid produced longer shoots. Addition of casein hydrolysate significantly reduced callus response.

Key words: Ascorbic acid, activated charcoal, casein hydrolysate, organogenesis, shoot regeneration

INTRODUCTION

Many of the chemicals used in tissue culture media are known to influence caullogenic responses of explants in *in vitro* culture. Some of these chemicals include metabolites such as purines and pyrimidines (Thorpe, 1980), vitamins (George and Ravishankar, 1996), complex nutrients (Artunduaga *et al*., 1989; Pierik, 1993), antioxidants (Kalinin *et al*., 1992) and miscellaneous additives such as activated charcoal (Dumas and Monteuuis, 1995).

Antioxidants may affect morphogenic processes by providing protection against oxidative stress and destructive action of constantly arising active oxygen forms (Leshem, 1988; Inze and Montagu, 1995). Addition of antioxidants to a tissue culture medium enhances the development of isolated cells and tissues (Kataeva and Butenko, 1983; Kalinin *et al*., 1992). In tomato, synthetic oxidant phenoxan and the natural antioxidants such as glutathione and ascorbic acid stimulate growth of tomato calli (Poleschuk and Gorbatenko, 1995). Antioxidants, such as ascorbic acid and citric acid have also been reported to promote explant survival (Siddiqui and Farooq, 1996). l-ascorbic acid (vitamin C) is synthesized within the plant cell from hexose sugars which is an antioxidant, redox buffer and an enzyme cofactor, so it has multiple roles in metabolism and in plant responses to abiotic stresses and pathogens (Ishikawa *et al*., 2006).

In addition to antioxidants, activated charcoal is also added to tissue culture media. Charcoal is known to (1) adsorb compounds secreted from cultured tissues or those already present in the medium that would otherwise inhibit growth and (2) prevent unwanted callus growth and promote morphogenesis, particularly embryogenesis and iii) induce root formation (George, 1996).

Another media additive that has been advantageously used to enhance organogenic response is casein hydrolysate. Casein hydrolysate is produced by hydrolysing the milk protein casein by acids or enzymes to produce complex mixture of amino acids that are less costly than the identified amino acid. Casein hydrolysate has been successfully used in tissue culture of several plant species one of them is *Pinus patula* (Malabadi and Staden van, 2006).

In our previous studies by Bhatia (2003), it has been demonstrated that sucrose, nutrient concentration, plant growth regulator, genotype and explants affect regeneration potential of tomato. To the best of our knowledge, there are no previous reports on the effect of casein hydrolysate and activated charcoal on morphogenesis of tomato.

The present study was conducted to evaluate the potential of improving shoot regeneration response including the quality of shoots regenerated from tomato cotyledons using ascorbic acid, casein hydrolysate and activated charcoal.

MATERIALS AND METHODS

The current study was carried out at the Central Queensland University, Rockhampton in the year 2004.

Culture media: MS Murashige and Skoog (1962) basal medium was supplemented with varying concentrations

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of activated charcoal, ascorbic acid and casein hydrolysate. All the media, including the control were supplemented with 15 μM zeatin and were solidified with 0.8% agar. The pH of the media was adjusted to 5.8 using 1 M NaOH or 0.25 M HCl and dispensed (5 mL per tube) into transparent plastic culture tubes (25×80 mm^2) prior to autoclaving at 1.05 kg cm^{-2} (103.5 kPa) and 121°C for 15 min. The media were cooled to 40°C before the lids were tightened to minimise condensation. Ascorbic acid was filter sterlised by using millipore® membrane filter. Measured volumes of filtered solutions were added to pre-cooled medium at ca. 40°C.

Explant: Seeds of 'Red Coat' cultivar of tomato (*Lycopersicon esculentum* cv. Red Coat) were obtained from 'Yates Vegetable Seeds Co. Ltd'. Milperra, Australia. The seeds were surface sterilized for 15 min with 1% sodium hypochlorite and rinsed with sterile water before being transferred to autoclavable transparent culture tubes (25×80 mm^2) containing 5 mL MS basal medium solidified with 0.8% agar (Sigma Co. Ltd., USA). The cotyledons were excised from one-week-old seedlings and the whole cotyledons were inoculated onto media containing varying concentrations of activated charcoal, ascorbic acid and casein hydrolysate. The explants were aseptically placed with the abaxial (lower) surface touching the medium before being incubated in a controlled environment room. The culture tubes in all experiments were placed in a controlled environment room which was maintained at 25±2°C at all times and with a light intensity of 38 μmol m^{-2} sec^{-1} provided by cool white fluorescent tubes (Sylvania Gro-Lux, Germany) for 16 h.

Experiment 1

Effect of activated charcoal on shoot organogenesis: Activated charcoal was added to the regeneration medium at the concentrations of 0, 1, 5 and 10 mg L^{-1}.

Experiment 2

Effects of ascorbic acid on shoot organogenesis: Ascorbic acid was added to regeneration medium to achieve concentrations of 0, 30, 60, 120, 240 and 480 μM.

Experiment 3

Effect of casein hydrolysate on shoot organogenesis: The effects of casein hydrolysate were tested at the concentrations of 0, 50, 100, 200, 500 and 1000 mg L^{-1}.

For all three experiments, 45 replications per treatment were used.

Observations: The inoculated cotyledons were assessed for frequency of explants showing organogenesis, callus formation, callus diameter, number of shoots produced per explant and the height of regenerated shoots after four weeks of inoculation.

Experimental design and statistical analysis: Culture tubes were arranged on the shelves of a controlled environment room according to a completely randomised design. Each tube had a single explant and was considered as an experimental unit.

Shoot regeneration and callus response data were analysed using GenStat (Anonymous, 2000) with a Generalised Linear Model (GLM) (McCullagh and Nelder, 1983) and a logit link function assuming a binomial error distribution (for shoot regeneration and callus response). The means presented are those predicted from the final model. Pair wise comparisons of means were conducted on the logit scale as per the GenStat RPAIR procedure. For the number of shoots produced per explant, shoot height and callus diameter, only those cultures in which shoot or callus was produced were included in the analysis. As such, these data were unbalanced and were analysed by GenStat using the AUNBALANCED (Payne, 2002) procedure. Pair wise comparison of means was performed using the Least Significant Difference (LSD) test at the 5% level. Graphs were prepared using Sigma Plot (SPSS Inc., USA). A protected LSD test was used to separate the means and LSD values are provided for those treatemetns that differed at $p<0.05$. For treatments that did not differ significantly ($p<0.05$), only p-values are provided.

RESULTS

Shoot response: Addition of activated charcoal did not affect shoot response at all concentrations except at 5 mg L^{-1} where it significantly ($p<0.05$) reduced shoot response (47%) compared the Control (69%) (Fig. 1A). Shoot response at 1 and 10 mg L^{-1} was similar to that in Control. Ascorbic acid and casein hydrolysate also did not have any significant ($p<0.05$) effect on shoot regeneration response (Fig. 1B, C). Morphological features of the shoots produced on the media containing activated charcoal, casein hydrolysate or ascorbic acid were also unaffected and the shoots appeared normal without observable nutrient deficiency symptoms.

Number of shoots per explant: Addition of activated charcoal, ascorbic acid or casein hydrolysate to the regeneration medium did not have any significant influence on the number of shoots produced per explant (Fig. 2A-C).

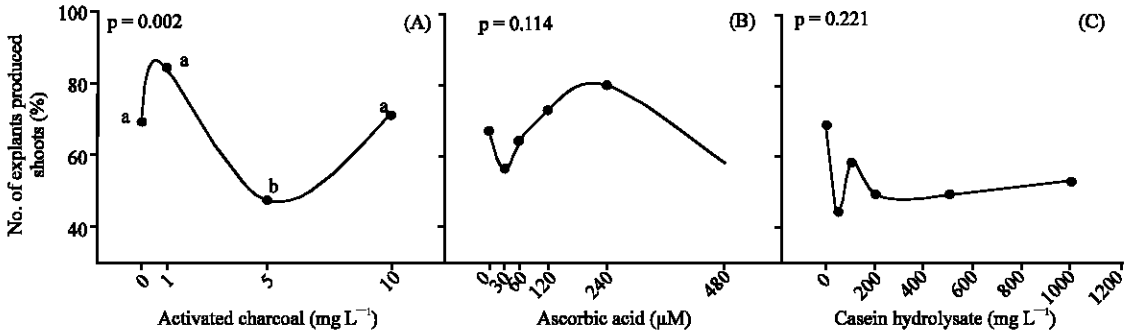

Fig. 1: Effects of activated charcoal, ascorbic acid and casein hydrolysate on shoot induction by cotyledonary explants of *Lycopersicon esculentum* cv. Red Coat (n = 45). LSD was not obtained as the data were analysed using generalised linear model (see Materials and Methods) and the means were compared using RPAIR procedure only if the p-value was <0.05. Means sharing the same superscripts do not differ significantly ($p<0.05$)

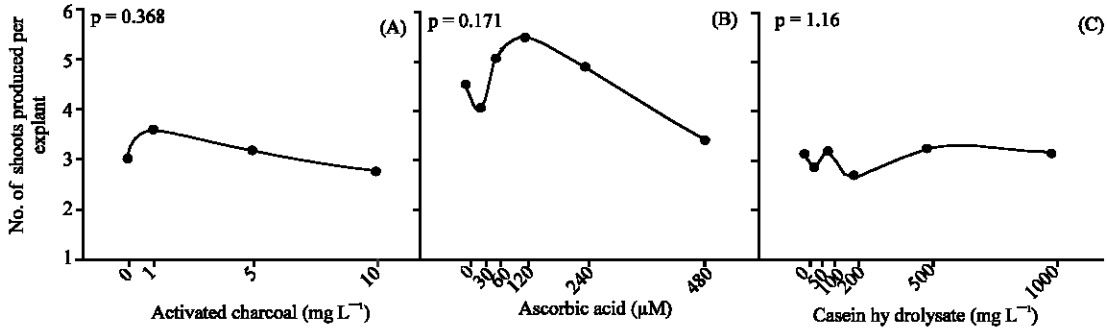

Fig. 2: Effects of activated charcoal, ascorbic acid and casein hydrolysate on the number of shoots produced per cotyledonary explant of *Lycopersicon esculentum* cv. Red Coat in four weeks after inoculation (n = 21-36)

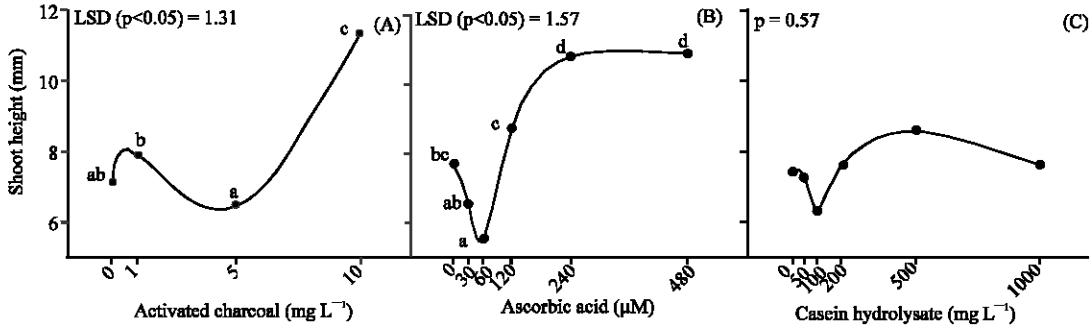

Fig. 3: Effects of activated charcoal, ascorbic acid and casein hydrolysate on the height of regenerated shoots developed from cotyledonary explant of *Lycopersicon esculentum* cv. Red Coat within four weeks after inoculation (n = 21-36). LSD is presented only if p-value was<0.05) and p-value is presented if it was not significant ($p>0.05$). Means sharing the same superscript do not differ significantly ($p<0.05$)

Shoot height: Shoot height in the control was similar to that of the treatments containing 1 or 5 mg L^{-1} activated charcoal. However, at 10 mg L^{-1} shoot height increased to 11.3 mm (Fig. 3A). Ascorbic acid did not improve shoot height until the concentrations were raised to 120 µM compared to control, but at 60 µM, the shoot height decreased significantly (Fig. 3B).

Casein hydrolysate had no significant ($p<0.05$) effect on the shoot height (Fig. 3C).

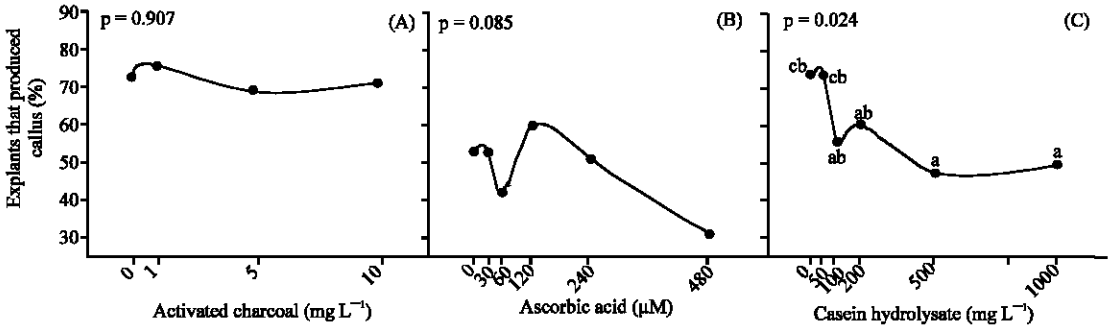

Fig. 4: Effects of activated charcoal, ascorbic acid and casein hydrolysate on the percentage of cotyledonary explants of *Lycopersicon esculentum* cv. Red Coat that produced callus within four weeks after inoculation (n = 45). Differences between the controls in three experiments are due to experimental error which is statistically non-significant. LSD is not obtained as data was analysed using generalised linear model (see Materials and Methods) and means were compared using RPAIR procedure only if the p-value was <0.05

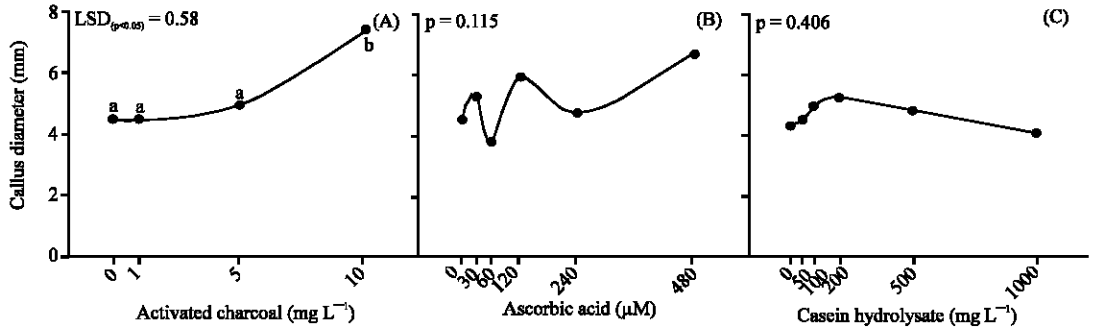

Fig. 5: Effects of activated charcoal, ascorbic acid and casein hydrolysate on diameter of the callus developed from cotyledonary explants of *Lycopersicon esculentum* cv. Red Coat in four weeks after inoculation (n = 14-33). LSD is presented only if the p-value was <0.05 and p-value is presented if p>0.05

Callus response: Callus induction was not affected by activated charcoal or ascorbic acid at any of the concentrations used (Fig. 4A, B). However, a significant reduction in callus response was observed with casein hydrolysate at concentrations >50 mg L^{-1} (Fig. 4C).

Callus diameter: Callus size at 10 mg L^{-1} (Fig. 5A) activated charcoal was significantly ($p<0.05$) higher than those at 1 or 5 mg L^{-1} or control. Ascorbic acid and casein hydrolysate had no effect on callus size across all concentrations (Fig. 5B, C).

DISCUSSION

The results of this study suggest that tomato does not require adsorbent (activated charcoal), antioxidant (ascorbic acid) or casein hydrolysate for morphogenesis. Activated charcoal is known to adsorb high concentrations of growth regulators (Constantin *et al*., 1977; Weatherhead *et al*., 1978) and may reduce shoot regenerative response. In the current study we observed a reduction in shoot response at 5 mg L^{-1} owing possibly to adsorption of plant growth regulators by activated charcoal. However, no reduction in shoot response was observed at 10 mg L^{-1}. At this concentration, activated charcoal would have adsorbed growth inhibitory substances along with the plant growth regulators. We hypothesis that these positive and negative effects of activated charcoal on shoot induction would have brought about shoot organogenic response to a level that was on par with the control. Shoot height followed similar trends and improvement in shoot height at the 10 mg L^{-1} activated charcoal was observed after reduction at 5 mg L^{-1} which further supports the above mentioned hypothesis. However, no phenolic or toxic substances were observed (as no colouring of the medium or browning of the medium could be detected). The non-phenolic inhibitory substances contained in

the medium either as medium impurities, or those produced in response to autoclaving. Release of phenols during tomato organogenesis is rare, nevertheless Rao *et al*. (1985) observed an accumulation of phenolic compounds in the suberised multilamellar wall during a 7-day culture period. These phenolic compounds comprised of 25% of the total monomers found in the tissue. The phenolic compounds were made up of vanillin (74.9%), p-coumaric acid (14.9%), p-hydroxy benzaldehyde (6.6%) and syringaldehyde (3.6%).

Ascorbic acid did not significantly affect any of the traits studied except shoot height indicating the absence of significant concentrations of oxidizing growth inhibitory substances produced in tomato *in vitro* culture. These results corroborate with those of Smirnov and Smirnova (1981) who found that ascorbic acid and glycine had little effect on shoot regeneration in tomato. Furthermore, Barar (1999) also did not observe any effect of ascorbic acid on shoot multiplication of certain genotypes of *Vigna radiata*. Contrary to these results, Shorning *et al*. (1999) reported an increased weight of hypocotyl explant derived callus. The beneficial effect of another antioxidant phenoxane is known to increase (1) apex-derived culture weight, (2) callus weight for cotyledonary explants, (3) leaf formation frequency and (4) root length and plantlet weight in tomato (Poleschuk and Gorbatenko, 1995). Similarly, the presence of the antioxidant phenoxane promoted shoot formation in tomato bud explants (Gorbatenko *et al*., 1994). A possible reason for the discrepancy between the above results and the current observations may be the use of different genotypes by the authors. The genotypic response to ascorbic acid has also been reported by Barar (l999) in *V. radiata* where the regeneration response to ascorbic acid was only observed in certain genotypes. However, in other genotypes, ascorbic acid abridged the regeneration response.

Similar to ascorbic acid, casein hydrolysate did not improve the morphogenesis. The only effect that the casein hydrolysate induced was in reducing the callus response. Casein hydrolysate is a source of calcium, phosphate, several microelements, vitamins and a mixture of up to 18 amino acids. Casein hydrolysate did not have significant influence on shoot regeneration. Possible reason for this could be the presence of optimum concentrations of phosphorus in the medium. Bister-Miel *et al*. (1988) reported that suspension cultures of *Cardamine pratensis* and *Silene alba* responded significantly to casein hydrolysate, when the medium contained sub-optimal concentrations of phosphorus. Casein hydrolysate significantly ($p = 0.024$) reduced the callus response. This finding is in agreement with that of Kim *et al*. (1987) who found the presence of casein hydrolysate in the medium inhibiting callus proliferation in *Ziziphus jujube*. In contrast to this finding, Artunduaga *et al*. (1989) showed the promotive activity of casein hydrolysate on callus proliferation in bermuda grass.

Conclusively, quality of regenerated shoots could be improved using activated charcoal and ascorbic acid. Casein hydrolysate can be effectively used to reduce callus response underneath the shoots, thus reducing the chances of somaclonal variation.

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