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Effect of Nutrient Media and Sucrose Concentration on Shoot Organogenesis in Tomato

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Abstract

The F_1 hybrid Red Coat is one of the most highly sought after cultivars of tomato in Australia and yields up to 7.5 kg/plant. An experiment was conducted to determine the optimal strength and type of growth medium and sucrose concentration for shoot organogenesis of the Red Coat cultivar using cotyledonary explants. Two basal growth media, viz. MS and Gamborg's B_5 at 0, ¼, ½, full or double strength along with sucrose concentrations of 0, 0.5, 1.5, 3 or 5%, were evaluated using 25 replications. The main effects of treatment and their mutual interactions were evaluated for the proportion of explants that produced callus and/or shoots, number of shoots produced per explant, callus diameter and shoot height. The explants failed to produce shoots in the absence of mineral nutrient. Only a small proportion of the explants (6% with B_5 and 3% with MS) regenerated shoots in the absence of sucrose. Lower sucrose concentrations (0.5-1.5%) along with full strength media were optimal for most of the traits studied. The B_5 medium outperformed MS medium for shoot organogenesis. For all the traits examined, significant differences in main effects ($P < 0.05$) and two-way interactions were detected, but no three-way interactions (medium type × medium concentration × sucrose concentration) were observed. Sucrose was found essential for the development of chlorophyll. Chlorophyll content increased with an increase in sucrose concentration up to 3% and decreased at 5% sucrose.

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Introduction

Tomato plant regeneration techniques are applied for recovery of plants from engineered or selected cells, the production of disease-free stock, germplasm storage, and fundamental investigations into plant developmental biology (Warren 1993). The current protocols applied for tomato transformation are based on shoot regeneration from leaf or cotyledon tissue co-cultivated with disarmed *Agrobacterium tumefaciens* harbouring binary vectors (Fillatti et al. 1987). The efficiency of such procedures is generally low (Frary and Earle 1996; Hamza and Chupeau 1993), as currently available regeneration systems are inefficient, and most of the transformed leaf or cotyledons cells do not develop into shoots (Peres et al. 2001). Despite numerous reports on plant regeneration in tomatoes (Bhatia et al. 2004; Fari et al. 1992; Izadpanah and Khosh-Khui 1992; Padliskikh and Yarmishin 1990), an efficient protocol for direct shoot regeneration, which could be used for recovery of genetically transformed plants and for mass clonal propagation, is still not available. The major emphasis of previous studies has been on the optimisation of plant growth regulators (Ancora and Sree-Ramulu 1981; Cassells 1979; Chen et al. 1999; Costa et al. 2000a, b; Padmanabhan et al. 1974; Venkatachalam et al. 2000; Zapata et al. 1981) with little attention being paid to other important factors that may influence shoot regeneration.

Sucrose is the most commonly used carbon source in heterotrophic and/or mixotrophic tissue culture. However, contradictory effects of supplied sucrose on plant metabolism have been reported. Exogenous supply of carbohydrate adversely affected growth and photosynthesis of certain *in vitro* grown plant species, such as *Chenopodium rubrum* (Schafer et al. 1992) and *Gardenia jasminoides* (Serret et al. 1996), while on the other hand stimulating effects of sugars on growth and photosynthesis have also been reported for *in vitro* cultured plantlets of tobacco (Paul and Stitt 1993; Ticha et al. 1998).

Sucrose is essential for the healthy growth of tomato cultures and most researchers have used it as the sole source of carbon (Chen et al. 1999; Compton and Veilleux 1988; 1991; Costa et al. 2000a, b; Venkatachalam et al. 2000). Locy (1995) reported that when tomato (*Lycopersicon esculentum*) callus or cell cultures were placed on media containing ribose as the sole carbon source, the tissues turned dark brown and ceased growth, and after about 60 days, bright green tissues (that were able to grow on ribose) emerged from about 3% of the brown necrotic callus pieces. The sucrose concentrations that induce good callus growth in culture media may not be optimal for morphogenesis, but lower or higher concentrations may be more effective (George 1993). Researchers have paid little attention to determine the best concentration of sucrose for morphogenesis in tomato.

The other chemical factor that has attracted little attention in tomato organogenesis is optimising the strength and type of plant growth medium (composition and optimum quantity). Most researchers have used MS full strength basal nutrient medium (Chandel and Katiyar 2000; Compton and Veilleux 1988; 1991; Kartha et al. 1976; Park et al. 2001) for direct shoot regeneration in tomato. The information on optimal strength and type of basal nutrient medium and its interaction with varying concentration of sucrose is currently not available.

The Red Coat, cultivar used in the current research is a leading cultivar of tomato and is marketed by the Yates Vegetable Seeds Co. Ltd. (Milperra, NSW, Australia). This cultivar matures in 10-12 weeks and yields up to 7.5 kg per plant. The plants of Red Coat have indeterminate habit and require low level trellising, therefore they are easier to cultivate and harvest than other indeterminate tomato cultivars. The Red Coat cv. also bears classic gourmet style fruits with the advantage of larger fruit size and earlier maturity compared to its competitors. Each fruit weighs 130-140g and is highly valued for its fruit qualities, such as firmness, shelf life, colour and flavour. Picking costs are much lower than those of other cultivars due to larger fruit size and earlier and more concentrated maturity (Jones 1999 - Personal communication). The Red Coat cultivar is also resistant to physiological disorders like grey wall and hollowing. The seed cost of Red Coat is A$200 for 1000 seeds. Huge demand exists for the seeds of the Red Coat cultivar, as it is grown for the export market (Thompson 2003 - Personal communication).

In an effort to develop a cost-effective protocol for mass propagation of the Red Coat cultivar, different strengths (0, ¼, ½, full or double strength) of two basal growth media, MS (Murashige and Skoog 1962) and Gamborg's B_5 (Gamborg et al. 1968) and varied concentrations of sucrose (0, 0.5, 1.5, 3 or 5%) were evaluated and organogenic and callus responses of cotyledon explants were quantified.

Materials and Methods

Explants

Seeds of tomato cv. Red Coat were surface sterilised for 15 minutes with 1% (v/v) sodium hypochlorite and rinsed with sterile water before being transferred to autoclavable transparent culture tubes (25 mm × 80 mm) containing 5 mL agar-water medium consisting of 0.8% agar (Sigma Chemical Company, St Louis, MO, USA). Cotyledons were excised from one-week old aseptically grown seedlings and the whole cotyledons were inoculated onto different media containing combinations of various sucrose concentrations and medium strengths. Explants were placed with the abaxial (lower) surface touching the medium before being incubated in a controlled environment room. The controlled environment room was maintained at 25±2℃ and 16 h photoperiod with a light intensity of 38 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Sylvania Gro-Lux, Germany).

Culture media

Either MS (Murashige and Skoog 1962) or Gamborg's B_5 (Gamborg et al. 1968) basal medium was used at 0, ¼, ½, full, or double strength (w/w) of the basal medium. Each of the above treatment combinations had sucrose concentrations of 0, 0.5, 1.5, 3 or 5%. 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 1M NaOH or 0.25 M HCl, and mechanically dispensed (5 mL per tube) into transparent plastic culture tubes (25 mm × 80 mm) prior to autoclaving at 1.05 kg cm^{-2} (103.5 kPa) and 121℃ for 15 minutes. To minimise condensation, the media were cooled to 40℃ before the lids were tightened.

Observations

Observations were recorded after four weeks of inoculation for frequency of explants showing organogenesis, callus formation, callus diameter, number of shoots produced per explant and shoot height.

Chlorophyll determination

Chlorophyll meter (SPAD 520, Minolta, Japan) readings of leaves of *in vitro* cultured shoots were recorded to determine the effect of treatments on the chlorophyll content of regenerated plants. First leaf was used to record the readings (five replications per treatment). The chlorophyll meter was calibrated with the actual chlorophyll content readings of the sap extracted from the first leaves using 80% acetone (leaves were ground in pestle and mortar, suspended in 80% acetone, centrifuged to 3000X and the supernatant was used to measure the chlorophyll), and measuring the absorption at 652 nm using a spectrophotometer (Harborne 1998).

Experimental design and statistical analysis

The treatments consisted of all possible combinations of the two types of media, five media strengths and five sugar concentrations. Twenty-five culture tubes were used per treatment combination and the tubes were incubated in a controlled environment room according to a completely randomised design. A single tube was considered as an experimental unit and each tube had a single explant. For the percentage of regeneration and callus response, only the treatments that showed some regeneration were included in the analysis. Data were analysed using GenStat® statistical software with a generalised linear model (GLM; McCullagh and Nelder 1983) assuming a binomial error and a logit link function (for shoot regeneration and callus response). The initial model included the effects of media, media concentration, sucrose concentration and the corresponding two-way and three-way interactions. The final model was determined by stepwise elimination of non-significant ($P > 0.05$) interactions. The means presented are those predicted from the final model. For shoot regeneration and callus response, pair-wise comparisons of means were conducted on the logit scale as per the GenStat RPAIR procedure. For number of shoots produced per explant, shoot height and callus diameter, only those cultures in which shoot or callus was produced were considered in the analysis. As such, these data were unbalanced and were analysed in GenStat using the procedure AUNBALANCED (Payne 2002). 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).

Results

Shoot regeneration

Explants failed to produce shoots in the medium lacking mineral elements but produced shoots at ¼-strength and above (Figure 1A, Plates 1, 2). The organogenic response increased with an increase in the medium strength up to the

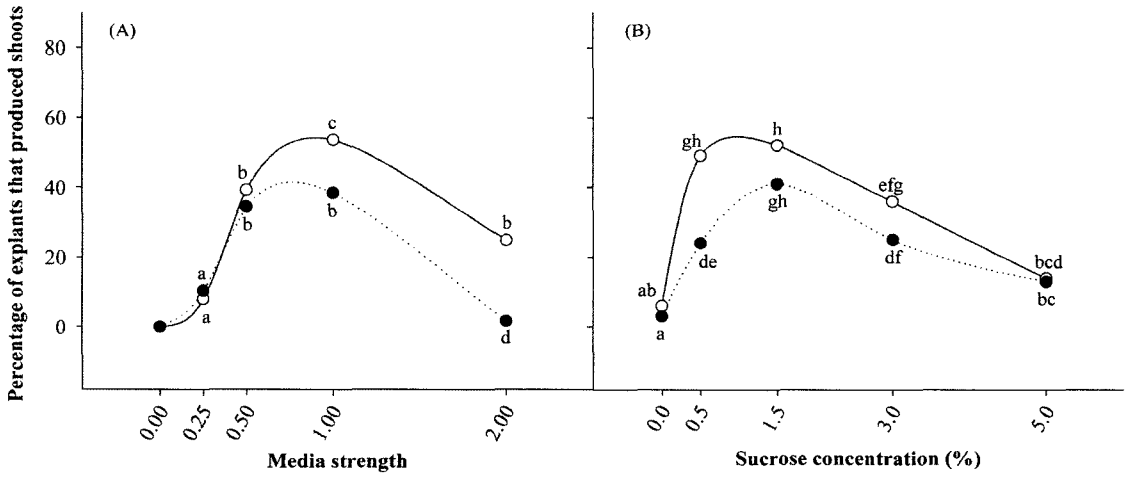

Figure 1. Effects of media, media strength (A; average of all sucrose concentrations) and sucrose concentrations (B; average of all media strengths) on the percentage of cotyledonary explants of cv. Red Coat that produced shoots in four weeks time. Open and closed circles represent B_5 and MS media, respectively. 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 significant (n = 125).

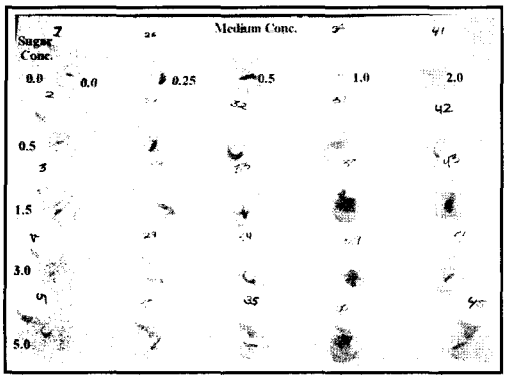

Plate 1. Morphogenic response in MS medium (the rows are sucrose concentrations and columns are medium strength) in four-weeks-old cultures of tomato cv. Red Coat.

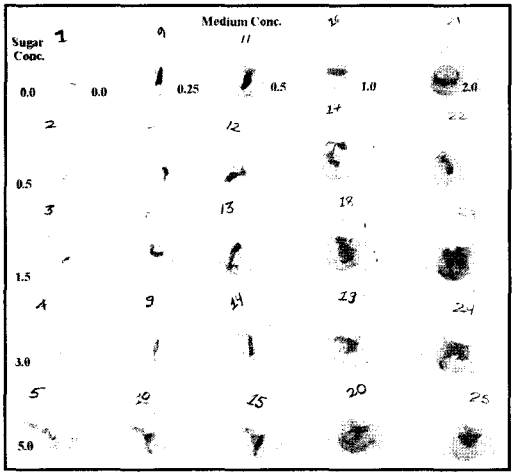

Plate 2. Morphogenic response in B_5 medium (the rows are sucrose concentrations and columns are medium strength) in four weeks old cultures of tomato cv. Red Coat.

full strength, but decreased significantly at the double strength and, this response was consistent in both types of media, indicating that the full strength was optimal for both media. In addition, it can be seen from Figure 1A that explants responded better in B_5 than in MS in terms of percentage of shoot regeneration when the media strength was 0.5 or above.

For the explants in B_5 medium, significant increase in shoot regeneration was observed when the sucrose concentration was increased from zero to 0.5%; but not when the concentration increased from 0.5% to 1.5%, the shoot regeneration decreased dramatically when the concentration was higher than 1.5%, thus the optimal sucrose concentration range with B_5 medium was 0.5% to 1.5% for shoot regeneration (Figure 1B). Similarly, for the explants in MS, shoot regeneration significantly ($P < 0.05$) increased with the increase in sucrose concentration up to 1.5%; and it significantly ($P < 0.05$) decreased at the concentration above 1.5%, hence the optimal sucrose concentration for MS is 1.5%. Only at 0.5% sucrose, explants performed better in B_5 than in MS; but no significant ($P < 0.05$) differences between the media were detected at other concentrations (Figure 1B).

Number of shoots

The number of shoots produced per explant increased with an increase in media concentration up to full strength in both media and decreased at double strength. Gamborg's B_5 medium was more favourable than MS at both full and double strengths, as evidenced by the significantly higher number of shoots produced per explant (Figure 2A).

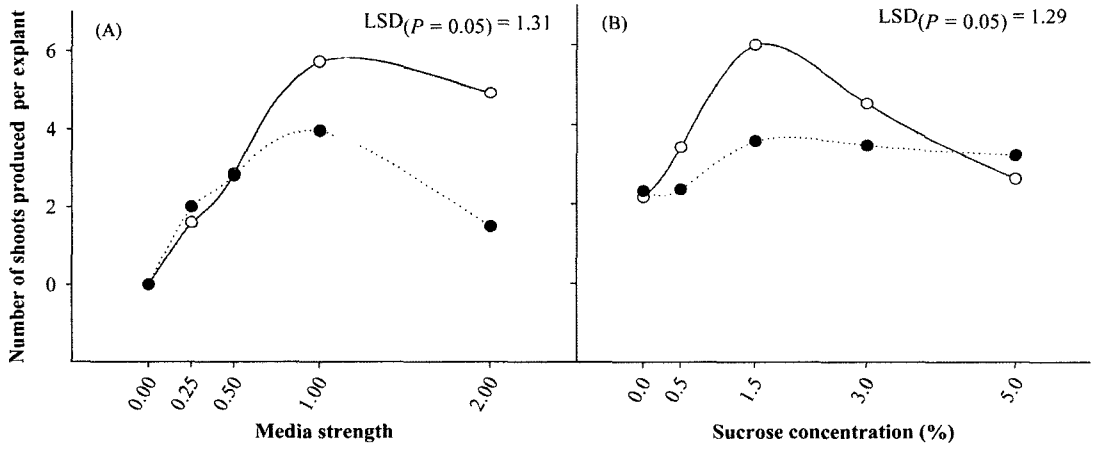

Figure 2. Effect of media, media strength (A; average of all sucrose concentrations, n = 1 - 42) and sucrose concentrations (B; average of all media strengths, n = 3-52) on the number of shoots produced per cotyledonary explants of cv. Red Coat in four weeks time. Open and closed circles represent B_5 and MS media, respectively.

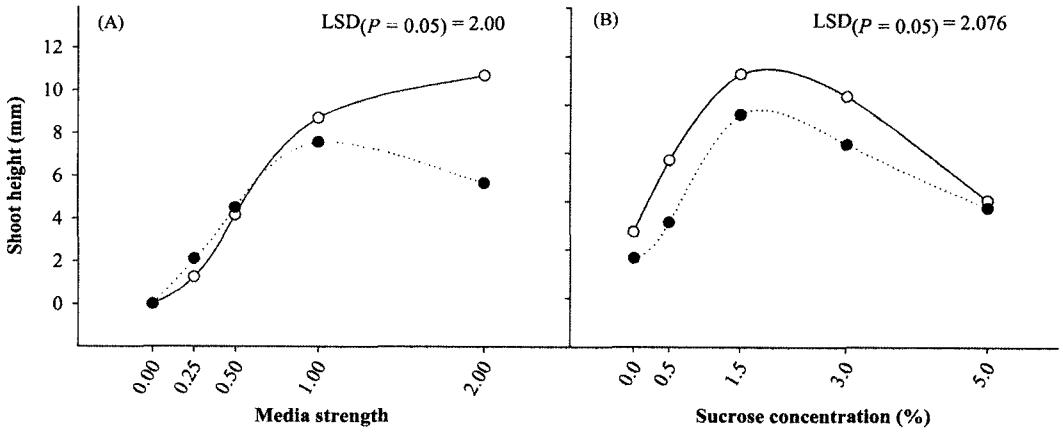

Figure 3. Effects of media, media strength (A; average of all sucrose concentrations, *n* = 3-66) and sucrose concentrations (B; average of all media strengths, *n* = 5-52) on the shoot height of the shoots produced from the cotyledonary explants of cv. Red Coat in four weeks time. Open and closed circles represent B_5 and MS media, respectively.

In terms of number of shoots produced per explants relative to sucrose concentration, explants performed best at 1.5% in B_5 media. However, in MS there was not any significant difference at 1.5 and 3% sucrose. Gamborg's B_5 medium produced a higher number of shoots than MS at most sucrose concentrations except at 0 and 5% (Figure 2B).

Shoot height

Shoot height of the regenerated plants increased with the increase in the media strength up to double strength in B_5 and up to full strength in MS, in which the shoot height decreased thereafter at double strength (Figure 3A). However, this reduction was not significant for MS medium. There was no significant difference in shoot height between the two media from 0 to full strength. However, at double strength, shoot height decreased for MS but kept increasing for the B_5 (Figure 3A). In both media, the tallest seedlings were observed at 1.5% (8.6 mm with B_5 and 6.4 mm with MS) and a further increase in sucrose resulted in a steady decline in shoot height (Figure 3B).

Callus regeneration

As for shoot regeneration, the explants also failed to develop callus in the absence of nutrient elements. There was a significant interaction between the media strength and sucrose concentration (Figure 4; it represents the combined results of MS and B_5). For quarter strength, maximum callus response occurred between 0.5 and 1.5% sucrose and decreased thereafter. No significant differences were observed in maximum callus induction among 0.5 to 5.0% sucrose with half strength of media. Generally, full strength

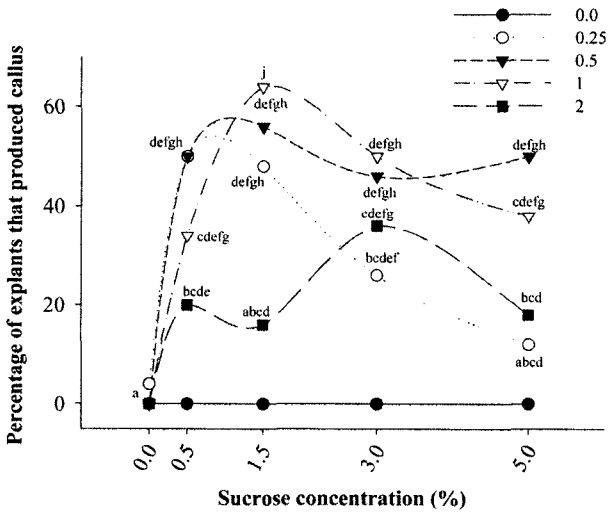

Figure 4. Effects of media strength and sucrose concentrations on the percentage of cotyledonary explants of cv. Red Coat that produced callus in four weeks time (*n* = 50). Data presented are averages of B_5 and MS media, as the interaction between medium type and concentration was significant. LSD 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 significant.

media at 1.5% sucrose produced a lot of callus and it decreased thereafter (Figure 4). For double strength media, maximum callus response (36%) occurred at 3% sucrose, and the response decreased at 5%.

Callus size

Callus diameter increased with the medium strength, and maximum callus diameter (5.26 mm) was observed at full

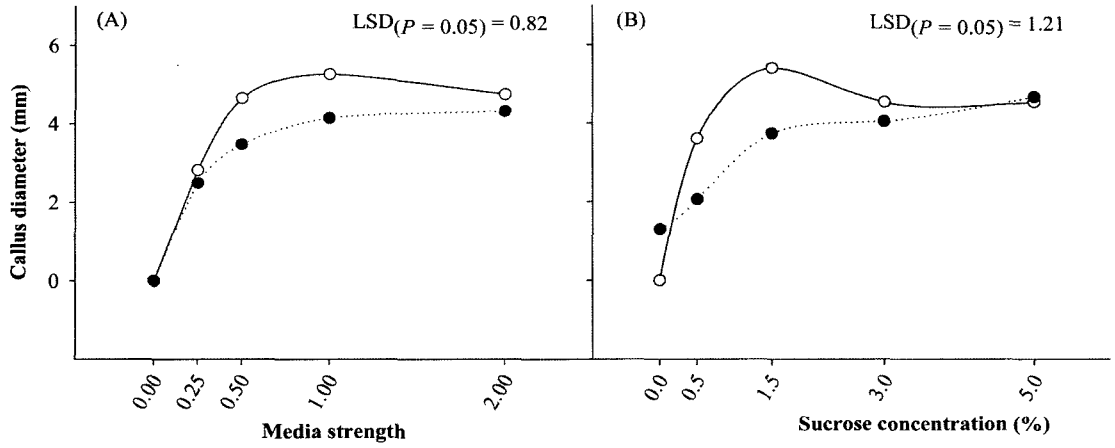

Figure 5. Effects of media, media strength (A; average of all sucrose concentrations, *n* = 7-64) and sucrose concentrations (B; average of all media strengths, *n* =1-55) on the callus diameter of the callus produced from the cotyledonary explants of cv. Red Coat in four weeks time. Open and closed circles represent B_5 and MS media, respectively.

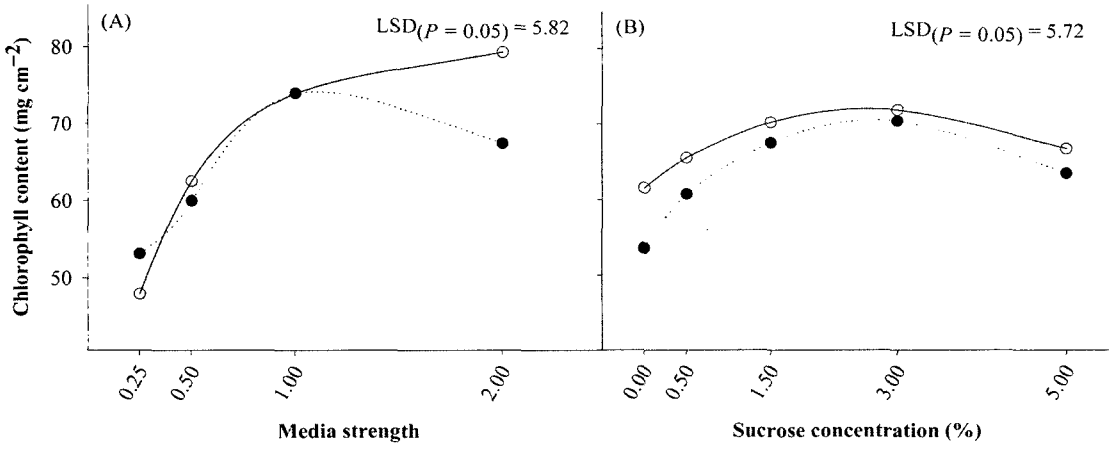

Figure 6. Effects of media, media strength (A; average of all sucrose concentrations) and sucrose concentrations (B; average of all media strengths) on the chlorophyll content of regenerated shoots of cv. Red Coat developed in four weeks time. Open and closed circles represent B_5 and MS media, respectively (*n* = 25).

strength for B_5 and at double strength for MS. (Figures 5A) but was not significantly different. Explants in both B_5 and MS media responded differently at various sucrose concentrations. Callus diameter increased with increase in sucrose concentration in MS (4.6 mm at 5%) whereas in B_5 it increased up to 1.5% (5.4 mm) and decreased at 3% or 5% sucrose (Figure 5B).

Chlorophyll content

Chlorophyll concentration in leaves of the *in vitro* cultured shoots increased with an increase in media strength up to full strength in MS (74 μg cm^{-2}) and double strength in B^5 (78.8 μg cm^{-2}) (Figure 6a). For MS, it decreased at double strength (66.9 μg cm^{-2}) (Figure 6A).

The presence of sucrose may be essential for the proper development of chlorophyll (Figure 6A). The chlorophyll content increased with an increase in sucrose concentrations up to 3% in both the MS and B_5 media and it decreased at 5% sucrose (Figure 6B).

Discussion

A general trend in this experiment reflects the requirement for lower sucrose concentration and moderate media strength (full strength) for organogenesis in tomato. While the presence of mineral nutrients was found necessary for shoot regeneration, shoots could be produced in the ab-

sence of sucrose at low nutrient concentrations. For the traits examined, there were significant effects of the main treatments i.e. medium type, medium concentration and sucrose concentration. Low-level interactions were also observed. However, no significant three-way interactions were observed amongst sucrose concentration, medium strength and type of medium for any of the traits studied.

Only a small number of explants produced shoots in the absence of sucrose. These explants became yellow and died (these were still included in the analysis if these were alive at the time of observation). Lack of sucrose in the culture medium may result in a substantial increase in RNAes and nuclease activities (Gallie et al. 2002) and these are linked to senescence of tomato tissues (Lers et al. 1998). This could have been a likely reason for the yellowing and death of the explants in the absence of sucrose in the current experiment.

Presence of sugar is required for explants to produce shoots, as sugar is converted into starch which accumulates in the target cells just before regeneration. Starch serves as a reserve of carbohydrates during meristem formation (Thorpe and Murashige 1968). Sugar affects morphogenesis also by changing the enzyme activity or expression of sugar-responsive genes (Rook and Bevan, 2003). In the present study the morphogenic response increased up to 1.5% of sucrose. Similarly Bhaskar *et al.* (2002) reported that 1.5% sucrose was optimum for multiple shoot development and early induction of protocorm-like bodies (PLBs) compared to 0, 3.0, 4.5, 6.0 and 7.5% sucrose in *Phalaenopsis* cv. Diana Pinky. Schnapp and Preece (1986) also obtained maximum shoot number (2.2) at 1 or 2% sucrose while micropropagating tomato through the axillary branching method.

Decrease in morphogenic response at concentrations above 1.5% could be explained by the regulatory role of the sugar. The possible reason for sugar influencing the organogenesis could be its interaction with the endogenous level of auxins. Calamar and Klerk (2002) reported an interaction between sucrose and auxin: increasing sucrose concentration shifted the dose-response curve of auxin to the right. Increase in sucrose concentration up to a certain level would increase the endogenous auxin levels. A further increase above 1.5% would have increased endogenous auxin concentrations to such an extent that suppressed shoot regeneration capacity of the explants. Inhibition of shoot regeneration at the higher sugar concentrations could also be due to increased ethylene production in the plant tissues (Meir et al. 1985). Increased osmotic stress associated with high sucrose content may also be a contributor to the reduced organogenic response.

Organogenesis in tomato appears to require moderate concentrations of mineral nutrients. Overall, the response was better in B_5 medium than in MS; possibly because B_5 contains lower N and higher PO_4 relative to the total ionic strength (phosphate/total ions), and such a requirement may be optimal for shoot organogenesis. However, total ionic strength of N and PO_4 $(N+PO_4)/\sum I$ {(nitrogen+phosphate)/total ions} is higher in B_5 (0.048) than in MS (0.036). It is probably due to these differences in N and P in the media, that B_5 induced better organogenic response, and produced higher number of shoots per explant and greater shoot length compared to MS medium. The difference in ionic strength in MS and B_5 medium causes differences in osmotic potential, thus affecting the culture response. Nutrient salts contribute from 20% to 50% of the osmotic potential of media while sugar contributes the rest. The total osmotic potential of a medium due to dissolved substances, can be estimated from ($\Psi s \approx \Psi s_{\text{macronutrients}} + \Psi s_{\text{sugar}}$; George 1993). Total ionic composition of MS medium is 95.8 mM in comparison to 61.9 mM in B_5 (George 1993) thus the comparative high osmotic potential of MS medium would have adversely effected the regeneration in tomato.

Furthermore, there was an interaction between the medium strength and the type of media for the organogenic response. Doubling the media strength reduced shoot regeneration capacity of the explants, although this reduction was smaller in B_5 than in MS. Since the basal MS contains much more mineral salts than B_5, doubling the media strength would have caused osmotic stress to the tissues leading to poor morphogenic response (George 1993). Another possible reason for the dissimilar response of the B_5 and MS medium could be due to differential change in the medium pH that could have affected the uptake of nutrients by tissues. Uptake of nutrients by tissues influences change in the medium pH, and sucrose-inverting capabilities of the invertase enzymes are maximal at the pH 3.6 to 4.7 (George 1993). A reduction in the medium pH causes hydrolysis of sucrose into glucose and fructose and hence influences the uptake of sugars. Dissimilar change in the pH in MS and B_5 medium would have resulted in divergent sugar uptake and hence different morphogenic responses between the two media. Plant tissues take up sugar molecules partly through passive permeation and partly through active uptake. Nutrients at various concentrations could compete with the sugar uptake. Gamborg et al. (1974) also explained that the uptake of inorganic ions can depend upon sugar concentrations in the tissue culture media. Since the B_5 medium has lower amounts of total nutrient elements (low ionic strength), it may cause less competition for sugar uptake compared to MS medium, thus resulting in efficient use of the sugar and better morphogenic response.

Data on chlorophyll content indicate that at least some sucrose is required for proper development of the chlorophyll. However, as the sucrose concentration increased, the chlorophyll development decreased at > 3% sucrose concentration in both B_5 and MS media. The same concentration of sucrose (1.5%) was also found to be the optimal for *in vitro* cloned papaya chlorophyll content in an experiment that tested sucrose concentrations ranging from 0% to 4% (Mondal et al. 1993).

The major effect of sucrose upon chlorophyll synthesis is shown at the stage when 5-aminolevulinic acid synthesis occurs. The activities of other enzymes involved in chlorophyll synthesis decrease at higher concentrations of sucrose, paralleling decreases in chlorophyll content (Pamplin and Chapman 1975).

The chlorophyll content increased with an increase in medium strength in B_5 medium, however for MS it was markedly reduced after the full strength. The chlorophyll development in plants has been shown to be directly proportional to the concentration of nitrate in the medium (Marek and Beranova 1989). The nitrate concentration in MS medium is 39.4 meq L^{-1} compared to 24.72 meq L^{-1} in B_5. In both media the chlorophyll content increased as the media strength raised up to the full strength; and thereafter, chlorophyll content increased in B_5 and declined in MS medium. The B_5 medium has almost half the nitrate concentration of MS, and by doubling the strength of B_5 the nitrate concentration would not have increased to a toxic level. Conversely, doubling the strength of MS would have increased the nitrate concentrations to a toxic level, and this may have interfered with chlorophyll synthesis, hence the noted reduction in chlorophyll concentration.

In summary, significant effects of the type and strength of the medium on organogenesis have been observed. Sucrose concentration also affected organogenesis significantly. Lower sucrose concentration (1.5%) along with full strength media, was optimal for organogenesis in Red Coat cultivar of tomato in terms of percentage explant response, number of shoots per explant and shoot height. The B_5 medium was found more suitable than MS for most of the traits studied. Sucrose was found essential for the development of chlorophyll. However, a higher concentration of sucrose (> 3%) adversely affected chlorophyll concentration.

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