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Comparative *in vitro* shoot organogenesis and plantlet regeneration in tomato genotypes

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ABSTRACT

A study was conducted for achieving high frequency regeneration in tomato genotypes as a pre-requisite for genetic transformation. Regeneration efficiencies were compared in five tomato genotypes using hypocotyl and cotyledon segments as explant source. Two cytokinins, namely BAP (0.0 to 3.0 mg l^{-1}) in combination with or without kinetin (0.5 and 1.0 mg l^{-1}) were used in Murashige and Skoog's medium. Irrespective of genotypes, 2.0 mg l^{-1} BAP supplementation gave the best response for both explants. The highest frequency shoot regeneration (96.6 and 92.2%) as well as the maximum number of shoots per explant (10.2 and 8.4) was obtained from the hypocotyl and cotyledon explants, respectively in genotype H-86. The cultures on this medium were green and showed good shoot bud organogenesis. The individual shootlets were separated and inoculated on growth regulator-free MS medium. After two weeks of root induction, the individual plantlets were transferred to glass jars filled with autoclavable polyproplylene (PP) caps filled with sterile peat : vermiculite (2:1). This hardening strategy lead to over 90.0% plant survival at greenhouse stage. The results suggested that of the two explants tested, hypocotyl segment was more responsive compared to cotyledon segment. The order of genotype response was H-86> H-24> DVRT-1> Sel-7> DVRT-2 with regards to shoot organogenesis and multiplication frequency.

Key words: *In vitro* regeneration, genotype, organogenesis, tomato.

INTRODUCTION

Tomato (*Solanum lycopersicon*, syn. *Lycopersicon esculentum* L.) belongs to family Solanaceae, is one of the most important vegetable crops. It is one of the crops which is amenable to physiological and cytogenetic investigations due to its ease of *in vitro* handling and genetic uniformity resulting from autogamy (Rick, 14). *In vitro* regeneration of cultivated tomato has been subjected to research on plant biology, and techniques have been developed for haploid and somatic hybrid production involving wild varieties (Wijbrandi *et al.*, 16). However, still techniques for large scale *in vitro* multiplication of commercially important cultivars is not available since the morphogenetic response is highly growth regulator-dependent or is also genotype-specific (Bhatia *et al.*, 1). *In vitro* regeneration in tomato may occur *via* direct organogenesis or somatic embryogenesis, but in most of the cultivated genotypes, the frequency of regeneration is much lower as compared to that in other members of the family like species of *Nicotiana Petunia* and *Solanum* (Gill *et al.*, 4). In the present investigation, an effort was made to standardize an *in vitro* organogenesis protocol and note the regeneration ability of some cultivated tomato genotypes.

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MATERIALS AND METHODS

Seeds of five tomato genotypes, namely, H-24 (Hisar Anmol), H-86 (Kashi Vishesh), Sel-7 (Hisar Arun), DVRT-1 (Kashi Amrit) and DVRT-2 (Kashi Anupam), were used for studying their *in vitro* regeneration potential. Freshly produced seeds of these genotypes maintained at the Indian Institute of Vegetable Research, Varanasi, Uttar Pradesh were used to avoid age difference. Healthy seeds were pre-sterilized by initially washing in running tap water and then treated with 20 mg l^{-1} 8-hydroxyquinoline citrate solution along with 0.50% Bavistin® (Carbendazim-BASF India) for 1h, washed with sterile double-distilled water and then transferred in 1% Cetrimide® solution for 10 min. For surface sterilization, the seeds were treated with 10% calcium hypochlorite (v/v) for 20 min with gentle shaking, and rinsed 5-6 times with sterile double-distilled water. Seeds were germinated *in vitro* on Murashige and Skoog medium (Muraghige and Skoog, 9) with pH adjusted to 5.8 and solidified with 7.0 g l^{-1} agar-agar in test tubes. The culture were maintained initially in dark for two days followed 16 h light and 8 h dark photoperiod, the light intensity being 60 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes. The culture room was maintained at constant temperature of 24 ± 2°C.

Shoot regeneration was studied using hypocotyl and cotyledonary explants (0.5-0.8 cm) excised from *in vitro* germinated 8-10-day-old seedlings and used for culture

on six different media combinations consisting of basal MS medium supplemented with BAP at 0.0, 0.5, 1.0, 2.0 and 3.0 mg l^{-1} in combination with or without kinetin (Kin) at 0.5 and 1.0 mg l^{-1}. Experiments were conducted using nine replicates with 10-12 explants per replicate. Data was recorded on shoot regeneration frequency. Shoots of about 20 mm size were dissected out of the cultures and transferred onto MS medium supplemented with different concentrations and combinations of GA_3 (0.0, 0.5 and 1.0 mg l^{-1}) and BAP/Kin (0.1 mg l^{-1}) for elongation. Micro-shoots (20 mm) were excised and individually cultured on different MS salt strength (1x, 1/2x, 1/4x and 1/8x salts) medium supplemented with different concentrations of IBA (0.0 to 0.5 mg l^{-1}).

Healthy *in vitro* raised plantlets with well-developed roots were removed from the culture tubes and washed thoroughly to remove the agar medium adhering to their roots. The roots were then dipped in a 2.0 g l^{-1} Bavistin® solution and the plantlets were transferred into small plastic cups/ bottles filled with autoclaved peat: vermiculite (2:1). The plantlets were irrigated with one quarter-strength MS salts solution. The cups/bottles were covered with transparent polythene bags, and placed in a culture room. After one week, the polythene bags were removed for 1 h; on the subsequent days, they were removed for longer periods of time (2, 4, 8, 12 h). Finally, the bags were removed and the plantlets were maintained in the culture room for another 8-10 days. The plantlets were then transferred into pots (15 cm dia.) containing a 2:1 mixture of soil and farmyard manure and the pots were kept in a glasshouse (26 ± 2 °C) where they grew and set fruits.

RESULTS AND DISCUSSION

The results of the present study showed that there were significant differences between explants and also genotypes for *in vitro* organogenesis and regeneration of plantlets. The specificities for auxin and cytokinin may be caused by different degradation rates of these substances in the tissues or in the medium, or by different aptitudes of the cells for their utilization and adaptation of these growth regulators. Hypocotyl and cotyledon explants excised from 8-10-day-old seedlings of H-24, H-86, DVRT-1, DVRT-2 and Sel-7 were evaluated for *in vitro* organogenesis. Callus formation was observable after one week in culture, while shoot buds could be seen after about 20 days in culture.

Genotypes showed significant difference on six media combinations. The hypocotyl and cotyledon derived regeneration varied significantly among the treatments used (Tables 1, 2). The maximum regeneration was observed on regeneration medium comprising MS medium in combination with 2.0 mg/l BAP in all the genotypes. The maximum number of shoots per explant (11.0 and 9.4) for hypocotyl and cotyledon respectively was observed for genotype H-86 followed by H-24. Regeneration response observed was media specific and genotype dependent too. Multiple shoots were regenerated from cotyledon segments after five weeks of culture. In case of other growth regulator combinations, some amount of callus was induced, while on hormone-free medium, explants only enlarged and did not form callus. It was evident that BAP was invariably required for shoot regeneration and the frequency of responding explants as well as number of shoots/explant was enhanced with addition of 2.0 mg l^{-1} BAP (Fig. 1). However, an increase in BAP concentration beyond 2.0 mg l^{-1} suppressed the frequency of shoot regeneration as well as the number of shoots per explant.

It was found that presence of BAP in the culture medium was essential for shoot regeneration. This finding is in conformity with the results reported by other workers. They reported that shoot regeneration from

Table 1. Effect of different combinations of BAP with or without kinetin on shoot regeneration from hypocotyl explants in different tomato genotypes.

Growth regulator conc. (mg l^{-1})	Genotype									
	H-86		H-24		Sel-7		DVRT-1		DVRT-2	
	A	B	A	B	A	B	A	B	A	B
MSO**	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
BAP 1.0	77.8^{b}	6.8^{cd}	81.7^{c}	3.7^{cb}	71.1^{c}	3.0^{c}	64.4^{b}	3.4^{b}	64.4^{b}	2.9^{d}
BAP 2.0	98.6^{d}	11.0^{e}	91.7^{d}	8.5^{d}	87.8^{d}	5.5^{d}	86.7^{e}	6.6^{d}	95.6^{d}	4.4^{e}
BAP 3.0	75.6^{b}	5.3^{b}	81.0^{c}	3.0^{b}	55.0^{b}	2.8^{c}	71.1^{c}	3.7^{b}	75.6^{c}	2.1^{c}
BAP 0.5 + Kin 0.5	85.6^{c}	7.7^{d}	75.0^{b}	4.4^{c}	73.3^{c}	2.9^{c}	72.2^{c}	4.5^{c}	90.0^{d}	1.9^{b}
BAP 0.5 + Kin 1.0	76.7^{b}	6.3^{c}	72.0^{b}	3.4^{b}	55.6^{b}	2.3^{b}	78.9^{d}	4.7^{c}	82.2^{c}	2.0^{bc}

**GR-free MS medium A = Frequency (%) of explants showing shoot regeneration; B = No. of shoots/ explant

Column values with similar values are non-significantly different with each other as separated per Duncan's multiple range test ($p<0.05$).

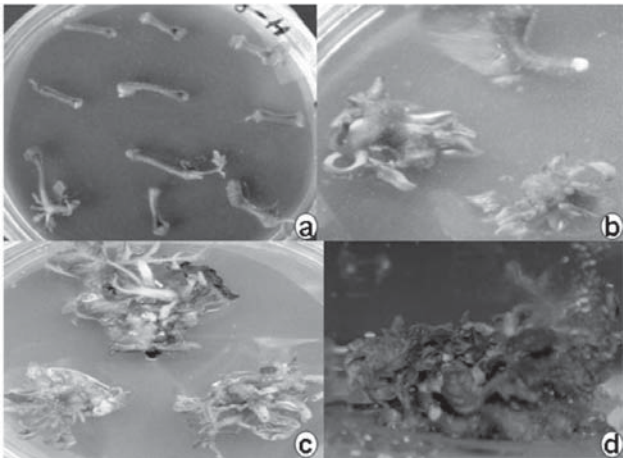

Fig. 1. Shoot regeneration from hypocotyl explants of tomato genotype H-86 in response to 2.0 mg l^{-1} BAP, (a)-(d) stages of multiple shoot regeneration.

tomato explants was dependent on the plant growth regulators, and in their absence, the explants only enlarged, swelled and then produced roots (Plastira and Perdikaris, 12; Moghaieb *et al.*, 8). However, these results are in contrast with the findings of Newman *et al.* (10) who observed regeneration through somatic embryogenesis and shoot organogenesis from hypocotyl sections of tomato cultured on hormone-free MS medium. Shoot regeneration from explants consisting of radicle, the hypocotyl and cotyledon devoid of meristem cultured on hormone-free MS medium has been also reported by Newman *et al.* (10) and Pozueta-Romero *et al.* (13). They suggested that nutrients and growth regulators supplied by the organized structure of the explant were responsible for the organogenesis on the hormone-free medium. In this study, shoot regeneration was observed on hypocotyl and cotyledon explants. Several workers have reported similar results using different explants hypocotyl and cotyledon (Gunay and Rao, 5; Moghaieb *et al.,* 8; Plastira and Perdikaris, 12), and leaf (Chandel and Katiyar, 2).

Among the different growth regulator combinations tested with both hypocotyl and cotyledon explants, 2.0 mg l^{-1} BAP was found to induce shoot regeneration from the highest frequency (%) and also produced the maximum number of shoots per explant. Similarly, Venkatachalam *et al.* (15) found 2.0 mg l^{-1} BAP to be the most effective in inducing multiple shoot regeneration from calli that were derived from hypocotyl explants by culturing them on MS medium supplemented with having 1.0 mg l^{-1} NAA and 0.1 mg l^{-1} kinetin. In contrast, Gunay and Rao (5) reported that cotyledon explants did not regenerate shoots when cultured on MS medium containing 2.0 mg l^{-1} BAP, while a combination of 2.0 mg l^{-1} BAP and 0.5 mg l^{-1} IAA elicited the highest response.

This experiment was planned to compare the shoot regeneration potential of the five tomato genotypes by culturing their hypocotyl and cotyledon explants on MS medium supplemented with 2.0 mg l^{-1} BAP. The data presented (Table 3) showed significant differences among genotypes as well as between hypocotyl and cotyledon explants for both shoot regeneration frequency (%) and number of shoots per explant. The genotype H-86 was found to be the best with respect to shoot regeneration frequency (96.6 and 92.2 % for hypocotyl and cotyledon explants, respectively) as well as number of shoots per explant (10.2 and 8.4 for hypocotyl and cotyledon explants, respectively), followed by H-24; the remaining three genotypes showed much lower regeneration potential than the two genotypes. Of all the genotypes studied, hypocotyl was appreciably much more responsive than cotyledon explants both in terms

Table 2. Effects of different concentrations of BAP in combination with Kin on shoot regeneration from cotyledon explants of different tomato genotypes.

Growth regulator conc. (mg l^{-1})	Genotype									
	H-86		H-24		Sel-7		Dvrt-1		Dvrt-2	
	A	B	A	B	A	B	A	B	A	B
MSO**	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
BAP 1.0	75.6^{b}	6.1^{c}	78.3^{d}	4.4^{c}	63.3^{c}	22^{c}	70.0^{b}	3.7^{c}	70.0^{b}	1.8^{b}
BAP 2.0	95.6^{e}	9.4^{e}	90.0^{e}	7.2^{d}	73.3^{d}	5.0^{d}	86.7^{d}	6.0^{f}	90.0^{d}	4.1^{f}
BAP 3.0	71.1^{b}	2.9^{b}	71.0^{c}	3.1^{b}	50.0^{b}	2.1^{c}	76.7^{c}	3.3^{b}	78.9^{c}	2.6^{d}
BAP 0.5 + Kin 0.5	87.8^{d}	7.2^{d}	71.7^{c}	3.7^{c}	60.0^{c}	2.2^{c}	73.3^{bc}	4.8^{e}	82.2^{c}	3.1^{e}
BAP 0.5 + Kin 1.0	81.1^{c}	6.2^{c}	63.3^{b}	3.0^{b}	52.2^{b}	1.7^{b}	78.9^{c}	4.0^{d}	81.1^{c}	2.2^{c}

A = Frequency (%) of explants showing shoot regeneration; B = No. of shoots/ explant.

Column values with similar values are non-significantly different with each other as separated per Duncan's multiple range test ($p<0.05$) .

Table 3. Effects of genotype and type of explant on frequency (%) of shoot regeneration and number of shoots per explant; the explants were cultured on MS medium supplemented with 2.0 mg l^{-1} BAP.

Genotype	Shoot organogenesis (%)		No. of shoots/ explant*	
	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon
H-24	91.1[b]	85.5[c]	8.0[d]	7.0[c]
H-86	96.6[c]	92.2[d]	10.2[e]	8.4[d]
Sel-7	84.4[a]	72.2[a]	5.4[b]	4.8[b]
DVRT-1	81.1[a]	78.9[b]	6.1[c]	5.3[b]
DVRT-2	88.8[b]	83.3[c]	4.3[a]	3.8[a]

*Means followed by different letters in their superscript are significantly different from each other (P<0.05); comparison by DMRT within columns only.

of frequency of responding explants as well as number of shoots regenerated per explant. Hence, further regeneration studies were carried out with H-86 genotype.

The differences observed for shoot regeneration potential among different genotypes are not surprising since regeneration in tomato is reported to be genotype-specific. Earlier, Moghaieb *et al.* (8) reported 50, 28 and 20% regeneration frequencies from hypocotyl explants of cultivars UC-9, Pontaroza and Zuishi, respectively. In the present study, hypocotyl explants from the five tomato genotypes showed regeneration of consistently higher number of shoots per explant from an appreciably higher proportion of the explants than did the cotyledon explants (Table 3). These findings are similar to those of Gunay and Rao (5), who observed shoot regeneration from 100% of the hypocotyl explants of tomato var. Karnataka, while only 60% of the cotyledon explants showed shoot regeneration. Similarly, Moghaieb *et al.* (8) reported that the meristematic ends of hypocotyl explants of tomato cultivar Pontaroza showed a much higher regeneration frequency as compared to the cotyledon explants. Similarly, Plastira and Perdikaris (12) found hypocotyl to be more responsive than cotyledon explants in terms of both shoot regeneration frequency and number of shoots per explant.

Shoots of 20 mm length, regenerated from explants of H-86, were excised and transferred to growth regulator-free MS medium or MS medium supplemented with GA_3 and BAP/Kin. After two weeks of culture, elongated shoots were obtained in all the cases. The effect of 0.5 mg l^{-1} GA_3 on shoot elongation was almost comparable to that of the GR-free MS medium (Figs. 2&3). Thus, an exogenous supply of GA_3 may not be required for elongation of shoots. In contrast, Venkatachalam *et al.* (15) reported that the shoots developed on MS medium containing 2.0 mg l^{-1} BAP formed rosette shoots and it was necessary to subculture them on MS medium containing a low level (0.1 mg l^{-1}) of BAP along with 1.0 mg l^{-1} GA_3 for proper shoot elongation. Novak and Maskova (11) reported that MS medium containing 0.25 mg l^{-1} GA_3 stimulated shoot and root development in shoot-tip cultures of tomato.

Shoots of about 20 mm, regenerated from explants of H-86 genotype, were cultured either on GR-free MS medium or on MS medium supplemented with different concentrations (0.0-0.5 mg l^{-1}) of IBA for root induction. The best response in terms of frequency (%) of rooted shoots and average root length was obtained on GR-free MS medium and on MS medium having 0.5 mg l^{-1} IBA (97.8%). Number of roots per shoot was the highest on the medium having 0.5 mg l^{-1} IBA, followed by that on GR-free MS medium (Fig. 2).

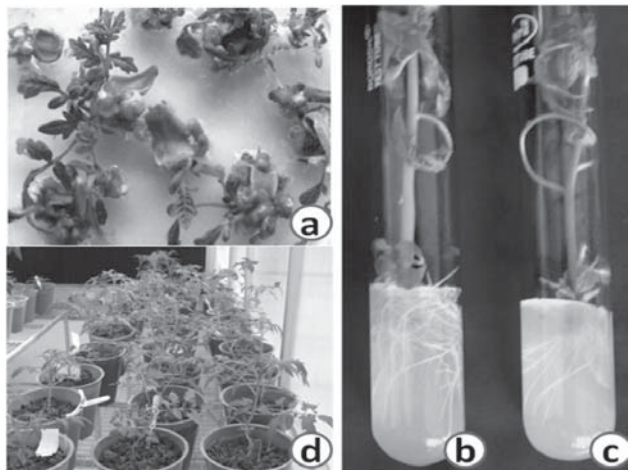

Fig. 2. Shoot multiplication and plantlet regeneration in tomato genotype H-86, (a) Multiple shoot regeneration from cotyledon explants on 2.0 mg l^{-1} BAP, (b) Elongation of *in vitro*-raised shoot MS + 0.5 mg l^{-1} GA_3, (c) GR-free MS medium, (d) Plantlets under hardening.

In the present study, GR-free MS medium and MS medium having 0.5 mg l^{-1} IBA were found to be the best for rooting of shoots. Similarly, Plastira and Perdikaris (12) used GR-free MS medium for rooting of shoots regenerated from leaf explants. But earlier workers, in general, have used an auxin to for rooting of shoots. Gunay and Rao (5) and Locy (7) reported that the regenerated shoots did not develop roots when transferred to GR-free MS medium, and that they rooted only when kept on medium supplemented with IAA. Similarly, Venkatachalam *et al.* (15) reported that shoots cultured on medium having 0.5 mg l^{-1} IBA gave the maximum number (10.7) of roots per shoot. Geetha *et al.* (3) used MS medium supplemented with 1.0 mg l^{-1} IBA for rooting of shoots regenerated from leaf calli. Kartha *et al.* (6) reported that shoots derived from shoot apical meristem cultures produced the maximum rooting frequency on MS medium supplemented with 1.0 mg l^{-1} IAA. The best response was obtained on GR-free MS medium having full-strength of salts; this medium yielded the highest frequency (100.0%) of rooted shoots, the maximum number (6.8) of roots per shoots and the highest average root (64.2 mm) and shoot (60.3 mm) lengths. A decrease in the concentration of macro- and micro-salts of MS medium significantly suppressed the percent of responding shoots, number of roots per responding shoot, and average root and shoot lengths (Table 4).

Table 4. Effect of IBA concentrations on induction of roots on *in vitro* grown shoots of H-86 tomato.

IBA conc. (mg l^{-1})	Rooting* (%)	No. of roots/shoot*	Av. root length (mm)*
MSO**	100.0^{d}	6.9^{c}	75.2^{c}
IBA 0.1	68.9^{c}	2.8^{a}	56.2^{b}
IBA 0.2	37.8^{a}	2.6^{a}	58.6^{b}
IBA 0.3	51.1^{b}	4.1^{b}	59.4^{b}
IBA 0.4	75.6^{c}	4.3^{b}	47.6^{a}
IBA 0.5	97.8^{d}	7.5^{d}	76.4^{c}

*Means followed by different letters in their superscript are significantly different from each other (P<0.05); comparison by DMRT within columns only.

**GR-free MS medium.

Thirty healthy *in vitro* raised plantlets from H-86 genotype were rooted and transferred to glass jars containing vermiculite and irrigated with one quarter-strength MS salts solution. Initially, the plants were maintained in a culture room and later were transferred to plastic pots (10 inch diameter) in glasshouse (Fig. 3). About 90% of the plantlets survived after hardening

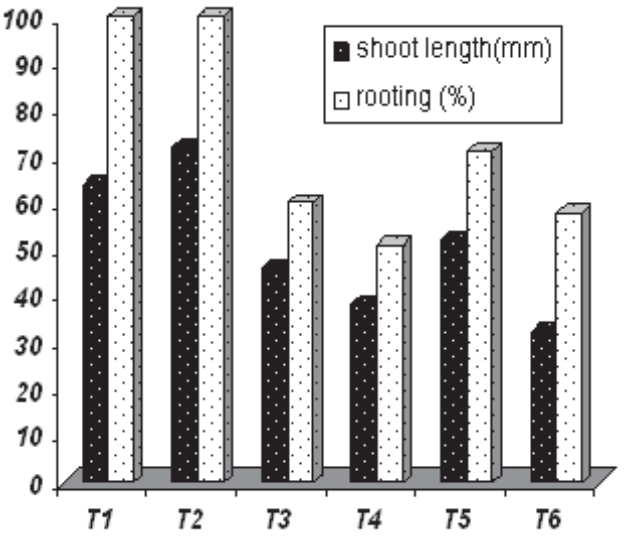

Fig. 3. Effects of different concentrations of GA_3 with or without BAP or Kin on shoot elongation in H-86 tomato (T1 = MSO; T2 = GA_3 0.5 mg l^{-1}; T3 = GA_3 0.5 mg l^{-1}+ BAP 0.1 mg l^{-1} ; T4 = GA_3 1.0 mg l^{-1}+ BAP 0.1 mg l^{-1} ; T5 = GA_3 0.5 mg l^{-1}+ Kin 0.1 mg l^{-1} ; T6 = GA_3 1.0 mg l^{-1} + Kin 0.1 mg l^{-1}).

and acclimatization. The *in vitro*-derived plants developed normal phenotype, flowered and showed normal fruit and seed set under glasshouse conditions. The results suggested that irrespective of genotypes, 2.0 mg l^{-1} BAP-supplemented MS medium gave the highest frequency of shoot regeneration as well as the maximum number of shoots per explant. Amongst genotypes, H-86 was most responsive with highest regeneration frequency (98.9 and 95.6%) and number of shoots per explant (11.0 and 9.4) for both hypocotyl and cotyledon explants, respectively. A comparative study of the five genotypes with the two explants on MS medium supplemented with 2.0 mg l^{-1} BAP the order of response was H-86 > H-24 > DVRT-1 > Sel-7 > DVRT-2 with hypocotyl explant excised from *in vitro* germinated seedlings.

In the present study, full-strength MS medium gave the best results in terms of frequency of rooted shoots(100%), number of roots per shoot (6.8), and root length (64.2 mm) as compared to ½, ¼ and $^{1}/_{8}$ strength MS salts medium (Table 5). Earlier, several workers (Venkatachalam *et al.,* 15; Chandel and Katiyar, 2) have suggested the use of half-strength MS salts medium for rooting of *in vitro*-proliferated shoots. The rooted plantlets were successfully hardened and transferred to glasshouse where they gave normal growth and fruiting. The protocol developed was then exploited for *Agrobacterium*-mediated genetic transformation.

Table 5. Effect of concentrations of MS salts on root induction in *in-vitro* grown shoots of H-86 tomato.

MSO/MSO modified medium	Frequency (%) of rooting*	Number of roots /shoot*	Average root length (mm)*	Average shoot length (mm)*
MSO**	100.0^{c}	6.8^{d}	64.2^{d}	60.3^{c}
MSO (½ salts)	75.6^{b}	3.5^{c}	44.8^{c}	40.4^{b}
MSO (¼ salts)	53.3^{a}	2.8^{b}	33.9^{b}	31.4^{a}
MSO ($^{1}/_{8}$ salts)	46.7^{a}	2.4^{a}	27.0^{a}	26.5^{a}

*Means followed by different letters in their superscript are significantly different from each other (P<0.05); comparison by DMRT within columns only.

**GR-free MS medium.

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