J Appl Phycol
DOI 10.1007/s10811-011-9717-9

Effect of seaweed extracts and plant growth regulators on high-frequency in vitro mass propagation of *Lycopersicon esculentum* L (tomato) through double cotyledonary nodal explant

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Received: 30 June 2011 / Revised and accepted: 21 August 2011

Abstract An efficient and reproducible two-step in vitro propagation system for tomato (*Lycopersicon esculentum*) was developed by using the combinations of seaweed biostimulant (*Gracilaria edulis* and *Sargassum wightii*) extracts and plant growth regulators (PGRs). Double cotyledonary nodal (DCN) explants of Co-3 cultivar were initially cultured on Murashige and Skoog (MS) and Gamborg's medium (B5) containing thidiazuron (TDZ) and 6-benzylaminopurine (BA); the best responding cytokinin was tested in combinations with different auxins (NAA, IAA and IBA), and seaweed extracts (*G. edulis* and *S. wightii*) of about basal MS medium +10–70% was used for shoot proliferation. The best organogenic culture response was obtained on MS medium fortified with 1.5 mg L^{-1} TDZ and 1.5 mg L^{-1} IBA. Up to 24 shoots per explants were formed at an optimal duration of exposure to 35 days. Mini shoots of about 3–4 cm were transferred to medium supplemented with MS + iP, MS + zeatin, MS + *G. edulis* and MS + *S. wightii* at different concentrations. High frequency of shoot elongation was observed in the medium supplemented with 30% *G. edulis* (15.2 cm), and profuse rooting was observed in the medium supplemented with 50% *S. wightii* of about 16.1 cm. Shoot elongation and rooting were observed in the medium supplemented with seaweed extracts. The plantlets were transferred to the plant growth chamber (70% of relative humidity and 9 light cycles) and maintained in it for a week, and then they were transferred to a greenhouse condition. The plant growth chamber to green house transferred plantlets showed an increase in the survival rate from 70 to 85%. Thus a two-step regeneration protocol was developed in this study with a combination of seaweed extracts and PGRs, which provides a basis for the production of transgenics with high frequency and survivability of tomato plants.

Keywords Tomato · Double cotyledonary nodal explants · *Sargassum wightii* · *Gracilaria edulis* · Rhodophyta · Phaeophyta

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Introduction

Tomato is a major vegetable and is cultivated in almost all parts of India. Tomato production and consumption has rapidly increased over the past two decades. Over 70% of Indian populations are directly engaged in agriculture (Bhatia et al. 2004). Various ecological imbalances such as global warming, increase in acidity of the soil texture, uneven rainfall and use of chemical fertilizers are factors that play a major role in the productivity of the crop. Due to modernization, use of chemical fertilizers has been widely adapted to increase the yield, and this leads to the various health hazards after consumption. Thus, there is a need to balance these effects by using natural resources for farming in positive manner.

Marine seaweed species are often regarded as an underutilized bioresource. Many have been used as a source of food, as industrial raw materials, and in therapeutic and botanical applications for centuries (Khan et al. 2009). Seaweed extracts have been applied as foliar spray and used in organic farming. These extracts are marketed as liquid

Published online: 16 September 2011

fertilizers and biostimulants, because they contain growth-promoting substances such as auxins and cytokinins that induce the shoot and root system consistently (Durand et al. 2003; Stirk et al. 2004). Presence of plant growth regulatory substances has been confirmed using chromatography (GCMS, LCMS) (Williams et al. 1981; Tay et al. 1985; Stirk et al. 1999). Furthermore, the wide range of growth responses induced by seaweed extracts imply the presence of more than one group of plant growth-promoting substances/hormones (Tay et al. 1985; Crouch and Van Staden 1993).

In vitro plant regeneration has been found to depend on many factors, of which most important are genotype, explant, composition of basic medium, growth regulators, gelling agent, light intensity and quality, photoperiod, temperature, cultivation vessels and vessel covers (Reed 1999). Several studies have reported adventitious regeneration in tomato dealing with induction of shoots on hypocotyls, apical meristem, cotyledons, stems petioles, leaves, anthers and inflorescences explants (Young et al. 1987; Branca et al. 1990; Compton and Veillux 1991). Gubis et al. (2004) reported that the frequency of adventitious shoot regeneration differed, depending on the type of explants and both the type and concentration of growth regulators added to the regeneration medium. Moreover, the adventitious shoot formation is based on the type of explant used and plays a major role in plant growth regulation (Pana et al. 2005). The development of a cost-effective and efficient protocol for mass propagation of high-quality seedlings via tomato tissue culture by using seaweed biostimulants instead of synthetic chemicals could help lower the price per seedling.

Besides commercially available auxins and cytokinins for in vitro propagation, an alternative can be used, namely seaweeds that promote the regeneration of plants. It has been reported that seaweed extracts improved the seed germination of table beet (Wilczek and Ng 1982; Khan et al. 2009) and several other horticultural crops and agricultural plants (Washington et al. 1999). Extracts from *Gracilaria gracilis*, *Cystoseira barbata* and *Codium tamentorum* have been tested for their effects on the germination of tomato, pepper and aubergine (Demir et al. 2006). Crouch and Van Staden (1992) reported that seaweed concentrate from *Ecklonia maxima* when applied as a soil drench significantly improved tomato seedlings. Plants treated with seaweed extracts showed an increase in total volume of root system and higher survival rate (Slavik 2005). It has been reported that the seaweed extracts also act as a defense mechanism against pests and fungal diseases. There is also progress towards the improvement of crops against viral, fungal, bacterial and pest diseases and environmental stresses by the introduction of genes that enhance tolerances against these biotic and abiotic stresses (Cluzet et al. 2004; Zhang and Ervin 2008). Research on the application of seaweeds against environmental stress factors is lacking. An efficient and reproducible regeneration system is essential for the stable genetic transformation improvement of the crop.

Mass propagation of tomato has been attempted through the use of various techniques, including shoot tip culture (Novak and Maskova 1979; Bhatia et al. 2004). Direct organogenesis from intact explants (Ichimura and Oda 1998; Bhatia et al. 2005) was established by using synthetic growth-promoting substances. In this paper we investigated the in vitro response of tomato towards seaweed extracts and plant growth regulators. Unlike chemical fertilizers, extracts derived from seaweeds are biodegradable, non-toxic, non-polluting and non-hazardous to humans, animals and birds.

Materials and methods

Gracilaria edulis and *Sargassum wightii* were collected from the coastal area of Rameswaram (9.28°N 79.3°E) during February 2011. The seaweeds were cleaned with sea water to remove impurities and epiphytes and then shade dried. The shade-dried algae were finely chopped and powdered. About 500 g of seaweeds were boiled in sterile distilled water for 50 min. Then the extracts were initially filtered through a muslin cloth and then filtered through Whatman no. 41 filter paper and stored at 4°C for further experimental studies.

Seed germination and initial culture establishment

The seeds of tomato (*Lycopersicon esculentum* L) cultivar Co-3 were obtained from the Tamil Nadu Agricultural University (TNAU). The seeds were washed under running tap water for 10 min, surface sterilized in 70% ethanol for 15 s and 0.1% mercuric chloride for 2 min and washed with three rinses in sterile distilled water. The surface-sterilized seeds were inoculated into an autoclaved 500-mL culture bottle containing a cotton bed with half-strength MS basal liquid medium (Murashige and Skoog 1962; Gamborg et al. 1968) and different concentrations (10–100%) of seaweed extract. Double cotyledonary nodal (DCN) explants excised from 3 to 6 days in vitro grown old seedlings were used as an explants source.

DCN explants were cultured on MS salts supplemented with B5 vitamins and various growth hormones and seaweed extracts. The nutrient medium consisted of major and minor salts, 3% sucrose as a carbon source and 0.8% agar (w/v). The effects of TDZ (0.5–2.5 mg L^{-1}) and BA (0.5–2.5 mg L^{-1}) were tested for the induction of multiple shoots from double cotyledonary nodal explants that were added to the MS medium in various concentrations. In

another experiment, the DCN explant was cultured on the medium containing 1–70% *G. edulis* and *S. wightii* extract for the induction of organogenesis. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 15 min. The cultured explants were maintained at 25±2°C under Philips fluorescent lamps (80 μmol photons m^{-2} s^{-1}) and a 16-h light/8-h dark cycle. The explants were subcultured every 10 days. There were ten replicates per treatment, and each experiment was repeated three times (n=30).

Multiplication of shoots

To attain high-frequency shoot proliferation the response to thidiazuron (TDZ) and 6-benzylaminopurine (BA) (0.5–2.5 mg L^{-1}) concentrations was individually tested for multiple shoot induction. To improve the frequency of shoot induction and shoot growth, the auxins were tested in combination with cytokinin. The best cytokinin concentration was used as a standard concentration, and it was tested against the various concentrations of NAA (0.3–1.8 mg L^{-1}), IAA (0.3–1.8 mg L^{-1}) and IBA (0.3–1.8 mg L^{-1}) in MS medium. In another set of experiments, the explants were cultured in MS medium containing seaweed extracts at different concentrations (10–70%). To maintain growth and vigor, cultures were subcultured on fresh medium every 10 days. Light and temperature conditions were maintained as described for the initial culture establishment system. The average numbers of shoots per explants were recorded at the third subculture, and the experiment was repeated thrice.

After proliferation, mini shoots of about 2–4 cm were excised from multiple shoots and cultured on MS medium supplemented with iP (0.4–2.0 mg L^{-1}), zeatin (0.4–2.0 mg L^{-1}) and 1.5–3% (w/v) sucrose and solidified with 0.8% (w/v) agar. The response towards these growth regulators was observed in a 1-week interval. In another experiment *G. edulis* and *S. wightii* extracts were tested at 10–70% concentrations in MS medium supplemented with carbon source and solidifying agent. Culture conditions were maintained as described previously. The average highest elongated shoots were recorded after 20 days of subculture. MS medium without any growth regulators is used as a control.

Root induction from elongated shoots

In order to induce high-frequency in vitro rooting, healthy elongated shoots were tested in basal MS medium (without any plant growth regulators) and auxins (NAA, IAA and IBA) at between 0.2 and 1.0 mg L^{-1}. The pH of the medium was adjusted to 5.75±2 before autoclaving for 15 min at 121°C. The cultures were incubated under a 16-h photoperiod at 25±2°C with 70% relative humidity and an irradiance of 80 μmol photons m^{-2} s^{-1}. The successfully rooted plantlets were transferred to a plant growth chamber.

Acclimatization of plantlets

Plantlets with well developed roots were removed from the culture bottles. The agar and other adherents from the in vitro cultured plantlets were washed in tap water and then hardened in plastic pots containing autoclaved vermiculite, sand and soil in a 1:1:2 ratio. Then the hardened plantlets were transferred to the plant growth chamber and grown for 7 days at 70% relative humidity and about 25°C. The plants were watered 2 days once in the growth chamber; the plantlets were covered with transparent plastic cups to avoid drying of young leaves. After 1 week, the plants were transferred to greenhouse conditions, and 25–30°C temperature was maintained. In the greenhouse, the plants were watered every 24 h, and humidity was maintained by water sprinkler.

Statistical analysis

The statistical significance of the data obtained from this study (mean number of shoots per DCN explants, mean shoot and root length and mean number of shoots per elongated shoots) was determined by one-way analysis of variance (ANOVA) (SPSS v. 17 for Windows 7).The mean values were compared by using Duncan's multiple range test (P<0.05).

Results

Seed germination and explants establishment

The primary part of the experiment is based on the preparation of contamination-free explants. Seeds of equal size were used for in vitro germination and germinated in liquid MS and G. *edulis* and *S. wightii* extracts in absorbent cotton in culture bottles. After 3 days of dark incubation at 25°C the highest seed germination (100%) was obtained in the culture bottle containing *G. edulis* and *S. wightii* extracts (Fig. 1). The control tomato seeds cultured in basal MS liquid medium only showed 80% germination. Double cotyledonary nodal explants were excised from the in vitro grown seedlings at 3–6 days and tested.

Effect of plant growth regulators and seaweeds on shoot initiation

DCN explants collected from the 4-day-old seedlings showed the best results (Fig. 2a). MS media containing BA and TDZ were tested individually for shoot induction.

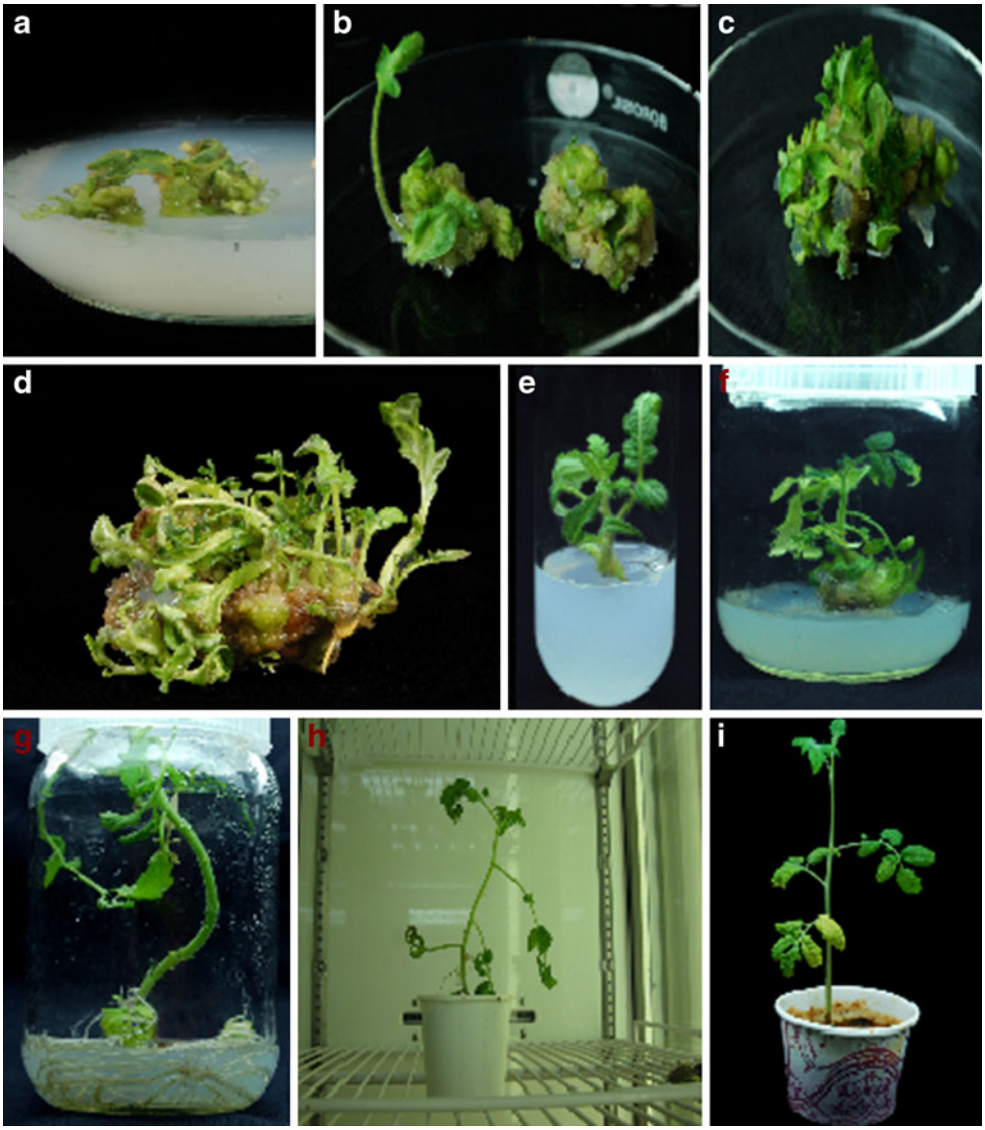

Fig. 1 Micropropagation of *Lycopersicon esculentum* L using double cotyledonary nodal explant. **a.** and **b.** Shoot bud regeneration on medium supplemented with 1.5 mg L^{-1} of TDZ and 1.5 mg L^{-1} of IBA after 20 days of subculture. **c.** Shoot bud proliferation on MS medium. **d.** Multiple shoot initiation and formation of mini shoots after four subcultures. **e.** Mini shoots cultured on MS medium containing 30% *G. edulis*. **f.** Shoot elongation and formation of basal callus at 3% of sucrose on medium fortified with 1.2 mg L^{-1} of iP. **g.** Rooting of in vitro regenerated shoots on MS medium supplemented with 50% of *S. wightii*. **h.** Hardened in vitro-derived plant in the plant growth chamber and maintained in it for 7 days. **i** Acclimatized plant outside the plant growth chamber

Medium supplementation with 2 mg L^{-1} BA and 1.5 mg L^{-1} TDZ gave the best response in multiple shoot induction (Fig. 2c). Shoot initiation was observed in the second subculture after 10 days. Multiple shoot induction frequency was increased in the third subculture. The highest number of shoots was observed in medium containing 1.5 mg L^{-1} TDZ and was 13.4 shoots per explants with a maximum shoot length of about 7.9 cm. BA-supplemented medium produced 11.9 shoots per explant at 2.0 mg L^{-1} concentration, and the highest shoot length of 7.2 cm was observed at 1.5 mg L^{-1} (Table 1).

In another set of experiments, *G. edulis* and *S. wightii* extracts at 10–70% concentration were used to evaluate the induction of multiple shoots. Medium supplemented with 30% *S. wightii* extract exhibited maximum number of shoots with about 4.8 shoots per explant. MS medium without any growth regulators (control) produced 2 shoots per explant, and the *G. edulis* extract-supplemented medium had a maximum of 3.5 shoots per explant at 30% extract concentration (Fig. 3). The highest mean shoot induction was in the medium supplemented with 1.5 mg L^{-1} TDZ, at which concentration it was tested against auxins (NAA, IAA and IBA) for the highest multiple shoot induction capability.

Individual TDZ-supplemented media produced a mean of 13.4 shoots per explant. Thus to test the capability of TDZ in combinations with auxins on the induction of multiple shoots, three auxins (NAA, IAA and IBA) at a concentration range of 0.3 to 1.8 mg L^{-1} were tested. TDZ in combination with NAA produced 11.4 shoots per explant, and the highest shoot length of about 3.5 cm was observed with 0.6 mg L^{-1} NAA. Subsequently, response of TDZ in combination with IAA was tested; the highest mean number of shoots (12.7 shoots/explant) was produced in the medium supplemented with 1.2 mg L^{-1} IAA and 1.5 mg L^{-1} TDZ. The highest mean shoot length of about

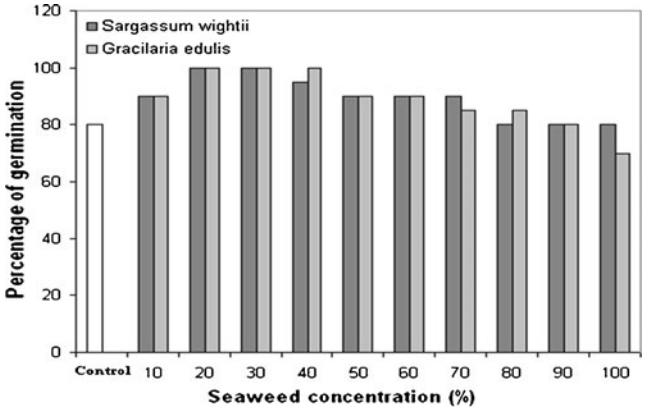

Fig. 2 Effect of MS basal medium, *Gracilaria edulis* and *Sargassum wightii* on in vitro seed germination of tomato. The medium was composed of MS salts and B5 vitamins, 3% sucrose and seaweeds at different concentrations. MS medium without any seaweed extracts is used as a control

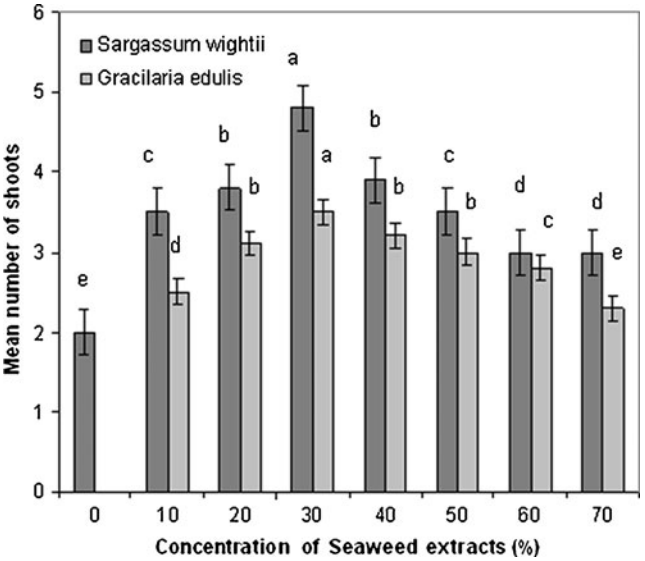

Fig. 3 Effect of *Sargassum wightii and Gracilaria edulis* on multiple shoot induction from double cotyledonary nodal explant. The medium was composed of MS salts and B5 vitamins, 3% sucrose and seaweeds at different concentrations. MS medium without any seaweed extracts is used as a control. *Error bars* = standard deviation of ten replicates, and each experiment was repeated thrice. Columns with different letters are significantly different at $P<0.05$. Data were recorded after 35 days of culture

3.3 cm was observed in the medium supplemented with 0.3 mg L^{-1} IAA in combination with TDZ. The frequency of multiple shoot induction increased to 23.5 shoots per explant in the medium with 1.5 mg L^{-1} TDZ and 1.5 mg L^{-1} IBA (Fig. 2d). Among the three auxins tested, IBA showed the best response compared to NAA and IAA. TDZ with IBA at 1.5 mg L^{-1} concentration responded well and recorded a maximum of 23.5 shoots per explant, with an average mean shoot length of about 4.1 cm (Table 2). The mini shoots of about 2–4 cm were excised and used for further experiments.

Table 1 Effect of plant growth regulators BA and TDZ on multiple shoot initiation from cotyledonary nodal explants of *Lycopersicon esculentum* L

	% of response	Mean number of shoots	Mean shoot length (cm)
BA (mg L^{-1})			
0.5	70	7.2 ± 0.13^{d}	3.7 ± 0.15^{b}
1.0	80	8.1 ± 0.23^{c}	$6.5\pm0.17b^{ab}$
1.5	90	11.0 ± 0.25^{b}	$\mathbf{7.2\pm0.53^{a}}$
2.0	**100**	$\mathbf{11.9\pm0.28^{a}}$	3.7 ± 0.15^{b}
2.5	90	10.9 ± 0.17^{b}	2.6 ± 0.16^{c}
TDZ (mg L^{-1})			
0.5	80	10.3 ± 0.30^{c}	5.2 ± 0.32^{c}
1.0	90	11.7 ± 0.15^{b}	6.4 ± 0.16^{b}
1.5	**100**	$\mathbf{13.4\pm0.16^{a}}$	$\mathbf{7.9\pm0.10^{a}}$
2.0	90	12.1 ± 0.10^{b}	6.8 ± 0.13^{b}
2.5	70	9.2 ± 0.13^{d}	$7.6\pm0.26a^{b}$

The shoot induction data was evaluated and scored after 35 days. DCN explants were cultured on MS medium fortified with different concentrations of BA and TDZ. Values represent the mean SE. Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test ($P<0.05$). Best results are indicated in bold

Effect of iP and zeatin in shoot elongation

Excised shoots from the multiple shoots were cultured in the MS medium supplemented with N^6-2-isopentenyl adenine (iP) and zeatin and tested for elongation in different concentrations (Fig. 2e). As formation of basal callus during elongation was noticed and as the formation of basal callus may reduce the survival rate of the plant during rooting, the experiment was designed to minimise the basal callus in elongated shoots. Three percent and 1.5% sucrose as carbon source were tested in iP and zeatin supplemented media. The shoot length recorded at 1.2 mg L^{-1} iP was 14.7 cm, and at 1.6 mg L^{-1} zeatin it was 12.5 cm (Table 3). Basal callus formation was observed when the medium was supplemented with 3% sucrose.

Induction of rooting from elongated shoots

The frequency and nature of the roots induced from elongated shoots varied with the type of auxin used. The shoots were blot dried on the sterile filter paper, and then they were transferred into the MS medium supplemented with auxins (NAA, IAA and IBA) at a concentration range of 0.2 to 1.0 mg L^{-1}. Rooting frequency was optimal, when the medium was supplemented with 0.4 mg L^{-1} IBA. Of the three auxins tested the IBA-supplemented medium produced a maximum of 17.4 roots per shoot and also

Table 2 Effect of combination of TDZ with NAA, IAA and IBA on multiple shoot initiation of *Lycopersicon esculentum* L c. v. Co-3

	% of response	Mean number of shoots	Mean shoot length (cm)
NAA+TDZ (mg L^{-1})			
0.3+1.5	90	9.8±0.13^{c}	2.8±0.13^{b}
0.6+1.5	**100**	**11.4±0.16^{a}**	**3.5±0.16^{a}**
0.9+1.5	100	10.5±0.17^{b}	2.5±0.16^{b}
1.2+1.5	90	9.7±0.15^{c}	2.2±0.32^{c}
1.5+1.5	90	8.1±0.10^{d}	1.8±0.13^{c}
1.8+1.5	80	7.9±0.10^{d}	1.3±0.15^{d}
IAA+TDZ (mg L^{-1})			
0.3+1.5	80	8.9±0.10^{e}	**3.6±0.16^{a}**
0.6+1.5	85	9.5±0.16^{d}	2.5±0.16^{b}
0.9+1.5	90	10.3±0.15^{d}	2.1±0.10^{c}
1.2+1.5	**100**	**12.7±0.15^{a}**	1.6±0.16^{d}
1.5+1.5	100	12.1±0.10^{ab}	1.2±0.13^{e}
1.8+1.5	90	10.6±0.16^{c}	0.9±0.36^{e}
IBA+TDZ (mg L^{-1})			
0.3+1.5	80	11.7±0.15^{f}	2.1±0.13^{d}
0.6+1.5	85	12.1±0.10^{e}	2.9±0.10^{c}
0.9+1.5	90	12.9±0.10^{d}	3.4±0.16^{b}
1.2+1.5	100	16.3±0.15^{b}	3.7±0.15^{b}
1.5+1.5	**100**	**23.5±0.16^{a}**	**4.1±0.10^{a}**
1.8+1.5	100	17.7±0.15^{c}	2.6±0.16^{c}

Media were supplemented with 1.5 mg L^{-1} of TDZ and different concentrations of auxins for the enhancement of multiple shoot induction. Data were collected after 5 weeks of culture. Values represent the mean SE. Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test ($P<0.05$). Best results are indicated in bold

produced the maximum root length of 15.2 cm (Table 4). The medium supplemented with 1.0 mg L^{-1} IAA produced 10.1 roots per shoot. Roots were very visible after a week of culture, and primary and secondary roots were produced after 10 days of culture. No basal callus formation in the rooting was observed.

Table 3 Effect of iP and zeatin on shoot elongation from in vitro mini shoots of *Lycopersicon esculentum* L

	Percentage of response	Mean shoot length (cm)	Basal callus formation at 3% sucrose	Basal callus formation at 1.5% sucrose
iP (mg L^{-1})				
0.4	80	10.1±0.10^{e}	++	++
0.8	90	11.6±0.16^{d}	++	− −
1.2	**100**	**14.7±0.15^{a}**	++	− −
1.6	100	13.5±0.16^{b}	++	− −
2.0	95	12.5±0.22^{c}	++	− −
Zeatin (mg L^{-1})				
0.4	85	9.1±0.52^{d}	++	− −
0.8	90	10.3±0.15^{c}	++	− −
1.2	100	11.7±0.15^{b}	++	− −
1.6	**100**	**12.5±0.16^{a}**	++	− −
2.0	100	11.4±0.16^{b}	++	− −

MS media were supplemented with iP and zeatin with 1.5% and 3% sucrose for shoot elongation. Shoot length and basal callus formation: ++ indicates that basal callus formed at the base of the shoot; − − indicates that no basal callus formation was recorded after 2 subcultures. Values represent the mean SE. Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test ($P<0.05$). Best results are indicated in bold

Effect of seaweed extracts on elongation and rooting of mini shoots

Shoots were excised from the high-frequency multiple shoots induced by TDZ and IBA at 1.5 mg L^{-1} concentration. Excised shoots were cultured in medium supplemented with *G. edulis* and *S. wightii* extracts at different concentrations (10–70%). The MS medium supplemented with 40% of *S. wightii* extract recorded a 14.6-cm shoot length, and the highest mean shoot length of 15.2 cm was observed in the medium supplemented with 30% *G. edulis* extract (Fig. 4). In another set of experiments 50% *S. wightii* concentrate supplemented to MS medium gave the best result of 16.1-cm root length, whereas 12.5 cm of root was observed in the medium supplemented with *G. edulis* concentrate (Fig. 5). Both shooting and rooting were observed in a single step, when the medium was supplemented with seaweed concentrate stimulants especially *G. edulis* and *S. wightii* (Fig. 2g). The average root length of about 14.01 cm was observed when the medium was supplemented with *S. wightii.* The combinations of TDZ × IBA and seaweed concentrates have produced healthy plants with no basal callus formation with high frequency regeneration during multiple shoot induction. The highest

Table 4 Effect of auxins on in vitro rhizogenesis

Concentrations	Percentage of root induction	Average no. of roots/shoot	Average no. of root length
MS basal medium	90	10.6±0.17	9.4±0.15
NAA (mg L^{-1})			
0.2	80	10.8±0.13^{e}	11.8±0.13^{d}
0.4	90	12.2±0.13^{d}	12.5±0.17^{c}
0.6	100	13.7±0.15^{c}	13.7±0.15^{b}
0.8	**100**	**15.3±0.15^{a}**	**14.1±0.10^{a}**
1.0	100	14.5±0.17^{b}	12.2±0.13^{c}
IAA (mg L^{-1})			
0.2	**100**	**15.8±0.13^{a}**	**13.8±0.13^{a}**
0.4	100	13.2±0.13^{b}	12.5±0.17^{b}
0.6	100	11.5±0.17^{c}	10.8±0.13^{c}
0.8	90	10.8±0.13^{d}	9.2±0.13^{d}
1.0	90	10.1±0.10^{e}	8.7±0.15^{e}
IBA (mg L^{-1})			
0.2	100	15.2±0.13^{c}	14.5±0.17^{b}
0.4	**100**	**17.4±0.16^{a}**	**15.2±0.13^{a}**
0.6	100	16.1±0.10^{b}	13.6±0.16^{c}
0.8	95	14.3±0.15^{d}	12.0±0.00^{d}
1.0	80	12.8±0.13^{e}	10.3±0.15^{e}

Data on number and length of roots produced at MS basal medium, NAA, IAA and IBA were scored after 20 days. Values represent the mean SE. Means followed by the same letter within columns are not significantly different, according to Duncan's multiple range test ($P<0.05$). Best results are indicated in bold

shoot length was recorded when the medium was supplemented with 40% *S. wightii* than iP.

Successfully rooted plants were hardened in plastic cups containing sterile soil and were transferred to a plant growth chamber. After 4 h, the young soft leaves had dried and some plants died in the growth chamber due to desiccation. So the plants were covered with the transparent plastic cups that reduced the drying of soft tissues (Fig. 2h). The plants were maintained in the growth chamber for a week, and then they

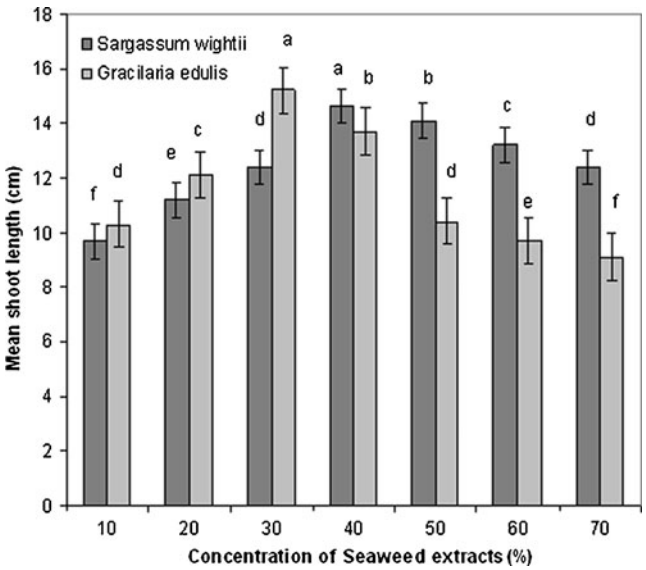

Fig. 4 Effect of *Sargassum wightii* and *Gracilaria edulis* on shoot elongation of microshoots from micropropagated culture. The medium was composed of MS salts and B5 vitamins, 3% sucrose and seaweeds at different concentrations. MS medium without any seaweed extracts is used as a control. *Error bars*=standard deviation of ten replicates, and each experiment was repeated thrice. Columns with different letters are significantly different at $P<0.05$. Data were recorded after two subcultures

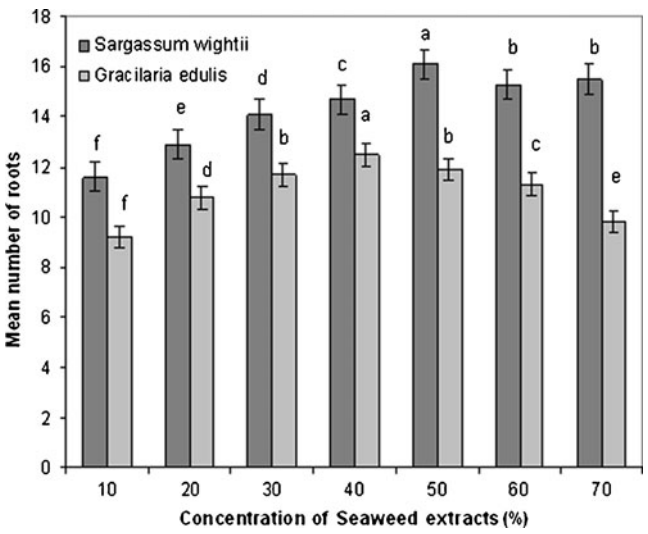

Fig. 5 Effect of *Sargassum wightii* and *Gracilaria edulis* on number of roots induced from elongated shoots. The medium was composed of MS salts and B5 vitamins, 3% sucrose and seaweeds at different concentrations. MS medium without any seaweed extracts is used as a control. *Error bars* = standard deviation of ten replicates, and each experiment was repeated thrice. Columns with different letters are significantly different at $P<0.05$. Data were recorded after 20 days of culture

were transferred to the greenhouse conditions. The survival rate increased from 70 to 85% when the plants were covered with plastic transparent cups. Some plants produced microroots on the shoots in the culture bottles. These microroots do not affect the survival rate of the plants, and these microroots dried as the plant grew in the greenhouse conditions. Thus all the surviving micropropagated hardened plants were free from external damages.

Discussion

Seaweed extracts have growth-stimulating activities and are used as biostimulants in natural crop protection. It was reported that the seaweed extracts have promising plant growth regulators such as auxins, cytokinins, gibberellins, betains and major macronutrients and micronutrients that help in promoting the growth of various vegetables, fruits and other crops (Miers and Perry 1986; Taylor et al. 1990; Blunden et al. 1991). This study showed that the seaweed extracts (*G. edulis* and *S. wightii*) enhance seed germination and induction of multiple shoots from DCN explants and induce the shooting and rooting of cultures in vitro.

Our findings clearly indicate that the *G. edulis* and *S. wightii* liquid extracts increase the seed germination percentage. Tomato seeds cultured in vitro in the MS medium supplemented with 20% seaweed extracts showed 100% germination, while 100% concentration showed the least germination. Our findings are in agreement with others (Kumar and Sahoo 2011; Xavier and Jesudass 2007) who have reported that 20% *S. wightii* extracts increase the percentage of seed germination. The seaweed liquid extracts have the capability to increase seed germination in vegetables and fruit crops (Hong et al. 2007). Also, tomato plants with *E. maxima* extract applied as a foliar spray set more flowers earlier than control plants. Seaweed extracts have also been shown to enhance both root–shoot ratio and biomass accumulation in tomato seedlings by stimulating growth (Crouch and Van Staden 1992).

In our study, we tested synthetic hormones and seaweed extracts for the induction of multiple shoots from DCN explants. Shoot organogenesis of some crops in tissue culture has been achieved recently using thidiazuron, a substituted phenyl urea compound with cytokinin activity (Baalma et al. 2008). Many reports state that TDZ induces shoot regeneration better than other cytokinins (Thomas 2003; Thomas and Puthur 2004; Husain et al. 2007). The cotyledonary explant of tomato which promoted the highest 6 shoots per explant was obtained on MS medium supplemented with 3.0 mg L^{-1} TDZ (Osman et al. 2010). In our study, TDZ produced a maximum of 14 shoots per DCN explant on MS medium supplemented with 1.5 mg L^{-1} TDZ, and 2 μM TDZ enhanced shoot bud development from cotyledonary nodal explant of mung bean (*Vigna radiata* L.) (Kumar et al. 2003).

Frequency was increased when the TDZ was tested in combinations with IBA at 1.5 mg L^{-1}; it produced 23.5 shoots per explant. But *G. edulis* extracts induced 3.5 shoots per explant at 30%. This activity was due to the presence of one or more plant regulating substances in the extracts. Beyond this 30%, shoot induction gradually decreased. Application of seaweed extracts enhanced shoot length and number of branches, root length and number of lateral roots at 20% treatment (Kumar and Sahoo 2011). Cocu et al. (2004) stated that combination of TDZ and IBA induced shoot regeneration from cotyledonary nodes in *Calendula officinalis*. It has been reported that the combination of NAA with BA or kinetin negatively affected the multiplication rate of the tomato compared with cytokinin used singly (Ishag et al. 2009). It also has been stated that the addition of NAA to medium containing cytokinin did not improve shoot multiplication rate. Earlier it was reported that the regeneration response of tomato to plant growth regulators (PGRs) has been highly genotype-specific. Type and concentration suitable for one genotype may not be optimal for others (Bhatia et al. 2004).

Mini shoots were cultured in MS medium containing iP and zeatin. Formation of basal callus was observed at the base of the elongated shoots, and these basal callus formations reduced the survival rate of the tomato plant. Therefore, various parameters were tested as sucrose concentration plays a major role in formation of basal callus. In vitro shoot elongation and rooting was observed in two different media. But mini shoots cultured on MS medium containing seaweed extracts promoted both shooting and rooting in the medium supplemented with 30% and 50% of *G. edulis* and *S. wightii*. Biostimulants in general are capable of increasing total volume of the root system (Slavik 2005). Thus seaweed products promote root growth and development (Jeannin et al. 1991). Our results clearly indicate that the seaweed extracts have growth-promoting activities at the single step compared to synthetic auxins tested. As per our knowledge this is the first report on establishment of in vitro mass propagation system for L. *esculentum* using seaweed extracts.

In conclusion in the present study we have established a high-frequency in vitro mass propagation system for *L. esculentum*. Higher frequency of micropropagation depends on type of explants, duration of collection of explants, concentration and combinations of PGRs, culture conditions and additives added to the medium. Apart from the synthetic auxins (NAA, IAA, IBA) and cytokinins (BA, TDZ, iP, zeatin), our results show that extracts of *G. edulis* and *S. wightii* play a significant role in shoot elongation and rooting of elongated shoots at concentrations of 30% and 50%, respectively. By using the naturally available marine resources, our protocol gave a better result than synthetic hormones tested. Hence it can be applied for commercial

production of tomatoes through micropropagation technique. Thus, seaweed extracts can be used as an alternative to synthetic growth regulators, and they reduce the chemical expenditure for tissue culture studies.

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