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BRIEF COMMUNICATION

Thidiazuron induced somatic embryogenesis and plant regeneration in *Capsicum annuum*

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Abstract

An efficient protocol of direct somatic embryogenesis (without involving intermediate callus) has been developed from stem segments and shoot tips of *Capsicum annuum* L. Explants were cultured on Murashige and Skoog (MS) medium supplemented with thidiazuron (TDZ). Among the various concentration of TDZ tested, 0.5 μM was proved to be best for induction of somatic embryos. Induction, maturation and germination were achieved on the same medium. The shoots developed from somatic embryos were transferred for rooting to MS medium supplemented with indole-3-butyric acid (IBA). All the regenerated plants with 85 % survival rate were normal with respect to morphology and growth characteristics.

Additional key words: chilli pepper, direct somatic embryogenesis, growth regulators, *in vitro*.

Capsicum annuum L. is an economically important crop plant and two main consumption types of pepper spice and vegetable are prevalent throughout the world. In order to facilitate development of plant biotechnology based cultivar improvement for this species, considerable effort has been devoted in developing and optimizing efficient *in vitro* regeneration protocols. Plant regeneration *via* organogenesis and somatic embryogenesis from diverse explants using different concentration and combinations of auxin and cytokinins has been described in pepper (Harini and Sita 1993, Fari and Andrasfalvy 1994, Steinitz *et al.* 1999, Ochoa Alejo and Ramirez-Malagon 2001). In spite of these reports, reproducible methods for routine propagation are not available and existing methods are not satisfactory.

Somatic embryogenesis, the production of bipolar structures from somatic cells is of considerable theoretical and practical importance because it can be used to combine efficient cloning with genetic modification (Sharp *et al.* 1980). *In vitro* somatic embryos can develop either from callus or directly from the explant without any intermediate callus stage. Since plant regeneration from callus cultures is often associated with genetic and cytological variations (Larkin and Scowcraft 1981) which are not always desirable, direct somatic embryogenesis has better applicability in the improvement of crops.

Thidiazuron (TDZ), a substituted phenyl urea (N-phenyl-1,2,3-thidiazol-5-yl urea) is a potent plant growth regulator which exhibit cytokinin like activity in various culture systems. TDZ was successfully applied to induce organogenesis from different explants such as wounded seedlings, intact seedlings, leaf, cotyledonary nodes and shoot tip of pepper (Szasz *et al.* 1995, Hyde and Phillips 1996, Dabauza and Pena 2001, Venkataiah *et al.* 2003) but none of these reports have used TDZ for the induction of somatic embryogenesis.

In this communication, we reported a high frequency regeneration system in chilli pepper through direct somatic embryogenesis from stem segment and shoot tip explants using TDZ, without involving callus phase.

Seeds of *Capsicum annuum* L. cv. Pusa Jwala were presoaked overnight and washed under running tap water for 30 min to remove adherent particles. The seeds were then immersed in 5 % (v/v) *Teepol* for 10 min, rinsed with sterile double distilled water. This was followed by surface sterilization with 0.1 % (m/v) $HgCl_2$ for 5 min and rinsed 5 times in sterile distilled water. The sterilized seeds were then placed onto basal Murashige and Skoog (1962) medium for germination. Stem segment (1 - 2 cm) excised from 10-d-old aseptic seedlings and shoot tip excised from 20-d-old seedlings were used as explants for induction of somatic embryogenesis.

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Abbreviations: IBA - indole-3-butyric acid; MS - Murashige and Skoog medium; TDZ - thidiazuron.
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Murashige and Skoog (MS) basal medium supplemented with 3 % (m/v) sucrose, and 1 % (m/v) agar was used during the study. The pH of the medium was adjusted to 5.8 with 1 M NaOH prior to autoclaving. All media were autoclaved at 121 °C for 15 min. MS basal medium was supplemented with different concentrations of TDZ (0.1, 0.3, 0.5, 0.8, 1.0, 1.5, 2.0 and 2.5 µM) for somatic embryo induction. All cultures were incubated in growth room at a temperature of 24 ± 2 °C with an irradiance of 50 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps for 16-h photoperiod. Induction, maturation and germination of somatic embryos into plantlets occurred on same TDZ supplemented medium. They were subcultured onto fresh medium three times after 2 week intervals. The percentage of explants producing somatic embryos and mean number of somatic embryos per explant were evaluated after 4 weeks of culture.

For root induction, the shoots (3 - 4 cm) developed from somatic embryos were harvested and transferred to MS medium supplemented with IBA at different concentrations (0.5, 1.0, 1.5 and 2.0 µM). Data were recorded on percentage of rooting, mean number of roots per shoot and root length after 2 weeks of culture.

Plantlets with well developed shoots and roots were removed from the culture medium, washed gently under running tap water and transferred to plastic pots containing sterile *Soilrite*. Potted plantlets were covered with transparent polythene membrane to ensure high humidity and watered every 3 d with half strength MS salt solution for 2 weeks. Polythene membranes were opened after 2 weeks in order to acclimatize plants to field conditions. After 4 weeks, acclimatized plants were transferred to pots containing normal garden soil and maintained in greenhouse under natural day length conditions.

All the experiments were repeated thrice and twenty replicates were employed for each treatment. The effect of different treatments was quantified and the data was analyzed using one way analysis of variance (*ANOVA*) and means were compared using the Duncan's multiple range test at 5 % level of significance.

The present findings demonstrate the induction of somatic embryos through stem segment or shoot tip explants cultured on various concentration of TDZ (0.1 - 2.5 µM). No somatic embryos were formed, when explants were cultured on hormone free MS medium. Stem segment explants was found more favorable in terms of induction of somatic embryos than shoot tip explant. For the induction of somatic embryogenesis, specificity in explant developmental stage of chilli pepper has also been emphasized by Harini and Sita (1993).

Globular somatic embryos were first observed 2 weeks after inoculation and they were white in colour. The initiation started from the base and later they appeared on the top of explant. These globular somatic embryos when subcultured 2 - 3 times onto fresh medium underwent orderly development through the heart, torpedo and cotyledonary stages (Fig. 1*A*,*B*,*C*). The frequency of explants producing somatic embryos and subsequent development into plantlets varied from 40 - 80 % in stem segment and 38 - 71 % in shoot tip explants on MS medium supplemented with various concentration of TDZ.

Among various concentrations of TDZ tested, best response in terms of number of somatic embryos per explants was observed on MS medium supplemented with 0.5 µM TDZ as it produced 21.9 ± 1.0 somatic embryos from stem segment and 15.7 ± 1.1 from shoot tip explants (Table 1). The frequency and number of somatic embryos per explant was found to gradually decline with increasing concentration of TDZ. The role of TDZ in the induction of somatic embryogenesis has also been reported in bamboo (Lin *et al.* 2004), cherry (Pesce and Rugini 2004) and *Sesbania drummondii* (Cheepala *et al*. 2004). These somatic embryos germinated on the same TDZ containing medium. The embryos germinated either attached to the mother tissue or isolated. The first signals of somatic embryos germination, namely the greening of cotyledons and radicle elongation were seen about one week of subculturing (Fig. 1*D*) which subsequently turned into plantlets having shoot and roots.

Table 1. Effect of TDZ on direct somatic embryogenesis from stem segment and shoot tip of *Capsicum annuum* L. after 4 weeks of culture. Values represent Means ± SE. Means within a column followed by the same letter are not significantly different by the Duncan's multiple range test at 5 % probability level.

TDZ [µM]	Explants producing somatic embryos [%]		Number of somatic embryos [explant^{-1}]		Number of somatic embryos producing plantlets	
	stem segment	shoot tip	stem segment	shoot tip	stem segment	shoot tip
0.1	40	45	9.3 ± 0.50^{e}	5.4 ± 0.32^{fg}	6.1 ± 0.21^{ef}	2.8 ± 0.30^{de}
0.3	65	60	14.0 ± 0.46^{cd}	8.9 ± 0.23^{cd}	10.4 ± 1.06^{c}	4.7 ± 0.31^{cd}
0.5	80	71	21.9 ± 1.01^{a}	15.7 ± 1.12^{a}	15.7 ± 0.90^{a}	11.8 ± 1.06^{a}
0.8	71	65	18.7 ± 0.85^{b}	12.5 ± 0.90^{b}	13.7 ± 0.64^{b}	8.8 ± 0.95^{b}
1.0	68	62	15.8 ± 0.92^{c}	10.6 ± 1.09^{bc}	11.4 ± 0.80^{c}	7.5 ± 0.54^{b}
1.5	60	58	13.3 ± 0.63^{d}	8.5 ± 0.30^{de}	8.6 ± 0.35^{d}	5.7 ± 0.30^{c}
2.0	49	45	11.0 ± 0.40^{e}	6.7 ± 0.34^{ef}	6.8 ± 0.20^{e}	4.5 ± 0.24^{cd}
2.5	40	38	7.1 ± 0.34^{f}	3.7 ± 0.24^{g}	4.7 ± 0.35^{d}	1.6 ± 0.29^{e}

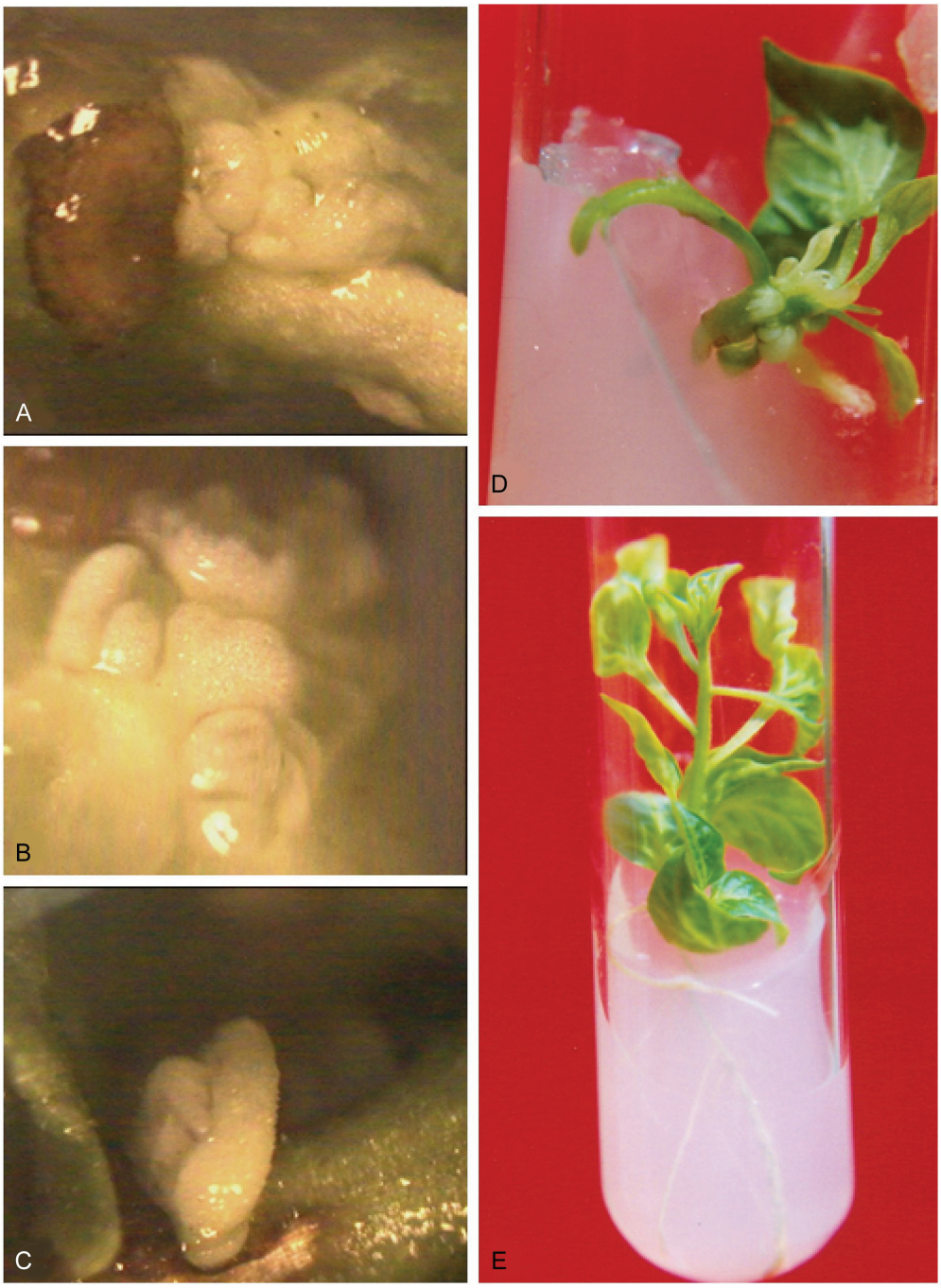

Fig. 1. Different stages of direct somatic embryogenesis in *C. annuum* on MS + TDZ (0.5 µM) and plantlet formation after 8 weeks of culture. *A* - cluster of somatic embryos; *B* - globular and heart shaped embryo; *C* - torpedo shaped embryo; *D* - germination of somatic embryos; *E* - rooted plantlet on MS + IBA (1.0 µM).

Induction and maturation of embryos is a two step process in many crops such as in *Fagopyrum* (Gumerova *et al.* 2003) and in niger (Venkateshan and Reddy 1996) but in present case induction, maturation and germination were achieved on the same medium. This method thus provides a one step process as in bamboo (Lin *et al.* 2004) and in *Astragalus* (Hou and Jia 2004).

Plantlets regenerated from somatic embryos were found to possess slender and weak roots. After removal of rooted portion, the shoots were transferred onto MS medium supplemented with various concentrations of IBA (0.5, 1.0, 1.5 and 2.0 µM). IBA (1.0 µM) was found to be

Table 2. Effect of IBA on root induction of shoots developed from somatic embryogenesis of *Capsicum annuum* L. in MS medium after 2 weeks of culture. Values represent Means ± SE. Means within a column followed by the same letter are not significantly different by the Duncan's multiple range test at 5 % probability level.

IBA [μM]	Rooting [%]	Number of roots [shoot^{-1}]	Root length [cm]
0.5	81	5.3 ± 0.34^{b}	2.4 ± 0.30^{bc}
1.0	90	6.4 ± 0.30^{a}	4.1 ± 0.40^{a}
1.5	78	4.2 ± 0.24^{c}	3.0 ± 0.23^{b}
2.0	70	2.5 ± 0.26^{d}	1.9 ± 0.11^{c}

the best for rhizogenesis as it produced 6.4 ± 0.3 roots in 90 % cultures after 2 weeks (Table 2, Fig. 1*E*). This is in accordance with earlier report on chilli (Agrawal *et al.* 1988).

Plantlets with 4 - 5 fully expanded leaves and well developed roots were acclimatized in *Soilrite* inside the growth room for 4 weeks and eventually established in soil. The percentage survival of the plantlets after transfer to *Soilrite* was 85 %. They grew well and attained maturity in greenhouse.

In conclusion, the present study describes an efficient protocol for somatic embryogenesis in chilli and has the potential to be used as a model system in an attempt to genetic transformations.

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