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# Micropropagation of Pepper (*Capsicum annuum* L.) Through *in vitro* Direct Organogenesis

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## ABSTRACT

An efficient procedure of *in vitro* plant regeneration through direct shoot bud induction was tested for different explants of *Capsicum annuum* L. The best performance was observed for cotyledons on MS medium containing 6 mg $L^{-1}$ 6-benzylaminopurine and 1 mg $L^{-1}$ indole-3-butyric acid. Regeneration for other types of explants (i.e., shoot tip, hypocotyl and root) did not show satisfactory results because the explants did not develop into normal shoots but instead developed into calli after 12 days of culture. Histochemical analysis showed that only the cotyledons revealed a direct induction of more teratological protuberances that arose around the cut end of the explants. Elongation of shoot buds was obtained on MS medium containing 1 mg $L^{-1}$ BAP + 0.5 mg $L^{-1}$ indole-3-butyric acid. Regenerated shoots rooted best on the same medium on which also elongation was realized. After hardening, the rooted plants were transferred to the greenhouse conditions where they grew, matured and flowered normally with a survival rate of 85%. We concluded that the present protocol can be efficiently used for mass propagation of sweet pepper.

**Key words:** *Capsicum annuum* L., mass propagation, micro-propagation, direct organogenesis, explants, regeneration, plant growth regulators

## INTRODUCTION

Peppers are the fruits of plants belonging to the genus *Capsicum* of the Nightshade family (Solanaceae). The genus consists of about 25 wild and 5 domesticated species (Sanatombi and Sharma, 2007). However, *Capsicum annuum* L. is considered as the economically most important species of the genus. The species includes both mild and pungent fruit types. In conventional systems seeds are generally utilized for multiplication and production. This method has some disadvantages such as: short viability period, low rate of germination, high risk of catching various diseases. In addition, pepper is sensitive to many pathogens and pests, including fungi, bacteria, viruses and nematodes and to extreme climatic conditions, especially temperature extremes, that are limiting factors for its production (Christopher and Rajam, 1994; Agrawal *et al*., 1988). In order to improve propagation of the commercial cultivars of these species and to meet the increasing demands for the crops, more reliable propagation approaches for mass multiplication are needed.

Tissue culture methods provide a way to asexually multiply pepper plants as the plants lack natural vegetative propagation.

Tissue culture aspects of the pepper plant have been well studied (Phillips and Hustenberger, 1985; Agrawal *et al*., 1988; Harini and Sita, 1993; Hyde and Phillips, 1996; Christopher and Rajam, 1996; Hussain *et al*., 1999). Several tissue culture techniques for micropropagation of pepper have been reported from different explants, including shoot tip (Christopher and Rajam, 1994), (rooted or unrooted) hypocotyls, leaf, stem, cotyledon, root, shoot tip and embryo (Agrawal *et al*., 1989) and induced somatic embryogenesis (Arous *et al*., 2001). However, many of the *in vitro* conditions set for a specific cultivar of the plant proved inappropriate for proper propagation of other cultivars. So, it is necessary to establish reliable regeneration systems for peppers, especially for genotypes developed for commercial purposes. Moreover, the comparative agronomic performance in terms of valuable traits, such as fruit or seed production, between regenerated and seed-derived plants has not been properly addressed, with only one study reporting the genetic and morphological variation of R0 and R1 pepper plants regenerated from *in vitro* tissue culture (Guadalupe *et al*., 2009).

In this study we developed an efficient protocol for *in vitro* micropropagation of an endemic pepper cultivar through direct organogenesis and for subsequent multiplication of the plantlets in the greenhouse.

## MATERIALS AND METHODS

The experiment was carried out at the Tissue Culture Laboratory of the Agricultural Biotechnology Research Institute of Iran in 2008. Seeds of a native cultivar of *Capsicum annuum* L. were obtained from the collection of the Agricultural Research Center.

**Plant material:** Seeds were washed with tap water for 5-10 min to remove surface contamination and then sterilized by immersing in 70% ethanol for 1 min with vigorous shaking followed by 20 min in 4% sodium hypochlorite containing one drop of Tween 20. The seeds were then rinsed three times with sterile distilled water in a laminar flow cabinet to remove minor amounts of disinfection liquid.

**Culture media and conditions:** For germinating, the seeds were cultured in mugs on 30 mL of standard Murashige and Skoog medium (1962) containing 3% (m/v) sucrose and 0.6% (m/v) agar. Cultures were incubated in a growth chamber at a temperature of 24°C and a 16 h photoperiod provided with a light intensity of 2000 lux using white fluorescent lamps. After 12 days, young seedlings having cotyledon leaves were used as the starting materials for direct organogenesis experiments.

**Shoot multiplication:** The explants (cotyledons, hypocotyls, roots or tips) were cultured on MS medium supplemented with BAP in seven different concentrations (0, 1.0, 2.0, 3.0, 4.0, 5.0, or 6.0 mg $L^{-1}$) combined with IBA in three different concentrations (0, 0.5, or 1 mg $L^{-1}$) to induce shoot formation. Sterilization was performed by autoclaving at 121°C for 20 min. pH was adjusted at 5.8 before adding 0.6% (m/v) agar. Explants were inoculated on polystyrene Petri dishes containing 30 mL of the desired medium. Each Petri dish contained 5 explants which were in contact with the medium surface. The cultures were incubated for 10 days in a growth chamber at a temperature of 24°C and with a 16 h photoperiod.

**Bud elongation:** Ten-days-old regenerated organogenesis structures were isolated from cotyledons and transferred into glass jars containing 50 mL of MS medium supplemented with 1 mg $L^{-1}$ BAP and 0.5 mg $L^{-1}$ IBA. After 20 days of subculturing, growth parameters including percentage of callogenesis, number of roots and number of buds were recorded and the effect of different shoot induction media was evaluated.

**Experimental design and statistical analysis:** The experiment was carried out in a completely randomized factorial design. Data were statistically analyzed using the SAS software (version 8). When the ANOVA indicated significant treatment effects (at 5 or 1%) based on the F-test, the Duncan's Multiple Range Test ($p = 0.05$) was used as a method to determine which treatments were statistically different from other treatments.

**Histological studies:** Histological staining was carried out to establish the ontogeny of explants containing shoot buds. Explants at different stages of their development (3, 8 and 12 days of regeneration) were fixed in FAA (formalin, acetic acid, absolute alcohol: 10, 5, 85; v/v) for 24 h. After dehydrating in ethanol (70, 95 and 100%) and xylene, textures were fixed in paraffin, sectioned with a microtome at 30 μm and then stained with methylene blue. The sections were prepared in a lam for observation under a light microscope.

**Rooting and acclimatization:** Most of the shoots in the elongation medium containing 1 mg $L^{-1}$ BAP plus 0.5 mg $L^{-1}$ IBA were rooted as well. In general, 4 weeks-old rooted plantlets were acclimatized and planted in pots containing sterilized peat mass and vermiculite (3:1 ratio). The pots were covered with a transparent beaker having a few holes in it and were frequently watered to keep high humidity in a phytotron during 10 days. Hardened plantlets were planted in a greenhouse set at a day temperature of 21°C, a night temperature of 19°C, a relative humidity of 85% and a day length of 12 h. Immediately after planting, the plantlets were irrigated and adequate soil moisture was maintained through daily watering. The proliferated plants showed 70-90% survival rate during hardening and acclimatization.

## RESULTS

Effects of source of the explant on *in vitro* regeneration were evaluated in terms of bud induction. The results showed significant differences among the four sources of explants. The explants from cotyledons gave the best result with respect to direct regeneration parameters (Table 1). The best response in terms of the percentage of explants that formed shoots was obtained around the cut surfaces of the cotyledons (Fig. 1a). Tip explants also showed some degrees of regeneration but none of the emergent buds developed into shoots and all showed callogenesis (Fig. 1b). The explants originated from hypocotyls and roots did not develop into buds and showed callogenesis. Only some degree of rhizogenesis was observed in root explants at the end of the regeneration period (Fig. 1c).

Direct bud formation was observed only in cotyledons cultured on MS medium supplemented with BAP (above 5 mg $L^{-1}$) and IBA (0.5 mg $L^{-1}$) which increased the percentage of organogenesis and advanced further development of the explants. Histochemical staining of the regenerated cotyledons also showed initial meristems and primordial formation in regions of bud formation after 8 to 10 days of culturing.

Table 1: Effect of growth regulators and explant type on callogenesis, number of roots and shoots per explant of *Capsicum annuum* L. in treatments with the best combination of growth regulators

| Growth regulators | Explant | Callogenesis (%) | No. of roots per explant | No. of shoots per explant |
|---|---|---|---|---|
| BAP 6 mg $L^{-1}$ + IBA 1 mg $L^{-1}$ | Cotyledon | 0.00c | 0.8a | 0.9a |
| BAP 6 mg $L^{-1}$ + IBA 1 mg $L^{-1}$ | Hypocotyl | 0.00c | 0.5b | 0.3bc |
| BAP 6 mg $L^{-1}$ + IBA 1 mg $L^{-1}$ | Root | 0.07a | 0.8a | 0.1c |
| BAP 6 mg $L^{-1}$ + IBA 1 mg $L^{-1}$ | Shoot tip | 0.02b | 0.8a | 0.5b |

Means with different letters are statistically significantly different at p = 0.05

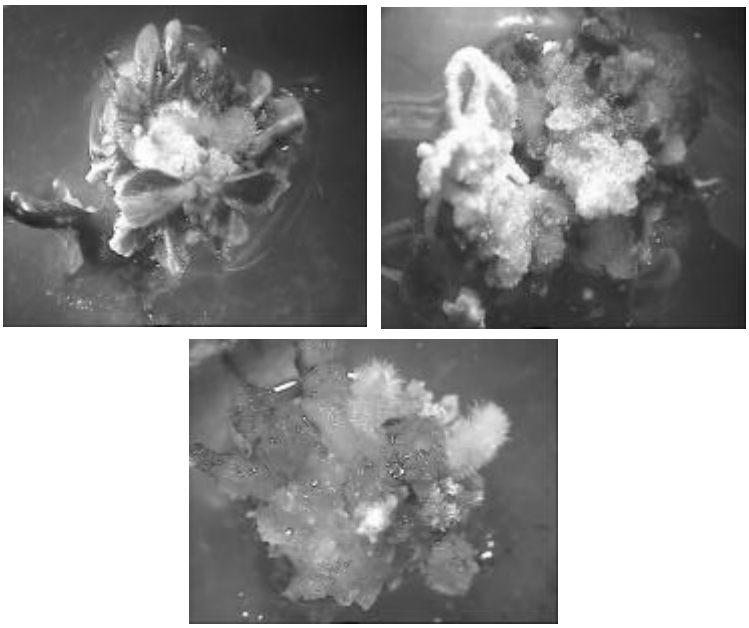

Fig. 1: Bud formation on different types of the explants. (a) shoots were grown around the cut surfaces of the cotyledons, (b) tip explants showed regeneration but none of the emergent buds developed into a shoot and all showed callogenesis and (c) root explants developed all into callus

Although, bud induction took place in either 5 or 6 mg $L^{-1}$ as well, further development of the buds into shoots was only observed in media supplemented with 6 mg $L^{-1}$ plus 1 mg $L^{-1}$ IBA. This treatment showed a higher percentage of direct organogenesis (Table 2) than the other treatments. Lowering the concentration of BAP in regeneration media below 5 mg $L^{-1}$ resulted in callogenesis and no bud induction. In the case of the tip explants low concentrations of BAP made the tips to develop into whole plants and no adventitious buds were observed.

After those 2 weeks the adventitious buds were transferred to an elongation medium with 1 mg BAP $L^{-1}$ plus 0.5 mg IBA $L^{-1}$. After 3 to 4 weeks of subculturing, shoot elongation took place followed by rhizogenesis (Fig. 2). So, elongation and root formation could be realized on one and the same medium.

Present results also showed that phenotypically there were no observable variations between the parents and *in vitro* propagated plants. The plantlets were hardened with a survival rate of more than 80% and then established well after planting them in a glasshouse (Fig. 3).

Table 2: Effect of combinations of growth regulators on callogenesis, number of roots and shoots per explant in *Capsicum annuum* L.

| BAP mg $L^{-1}$ | IBA mg $L^{-1}$ | Callogenesis (%) | No. of roots per explant | No. of shoots per explant |
|---|---|---|---|---|
| 0 | 0.0 | 0.49ab | 0.71d | 0.0d |
| 0 | 0.5 | 0.64a | 0.75d | 0.0d |
| 0 | 1.0 | 0.32bc | 0.83bcd | 0.0d |
| 1 | 0.0 | 0.21bc | 0.78d | 0.0d |
| 1 | 0.5 | 0.25bc | 0.76d | 0.0d |
| 1 | 1.0 | 0.01abc | 0.76d | 0.0d |
| 2 | 0.0 | 0.02c | 0.84bc | 0.0d |
| 2 | 0.5 | 0.09c | 0.86bc | 0.0d |
| 2 | 1.0 | 0.01c | 0.82bc | 0.0d |
| 3 | 0.0 | 0.21bc | 0.85bc | 0.0d |
| 3 | 0.5 | 0.01c | 0.83bc | 0.0d |
| 3 | 1.0 | 0.15bc | 0.83bc | 0.0d |
| 4 | 0.0 | 0.23bc | 1.06a | 0.0d |
| 4 | 0.5 | 0.14bc | 1.01a | 0.0d |
| 4 | 1.0 | 0.27bc | 0.90b | 0.0d |
| 5 | 0.0 | 0.12bc | 0.71d | 34.50c |
| 5 | 0.5 | 0.02c | 0.71d | 30.70c |
| 5 | 1.0 | 0.45ab | 0.71d | 28.60c |
| 6 | 0.0 | 0.09c | 0.71d | 84.30b |
| 6 | 0.5 | 0.01c | 0.71d | 85.50b |
| 6 | 1.0 | 0.22bc | 0.71d | 90.90a |

Means with different letters are statistically significantly different at p = 0.05

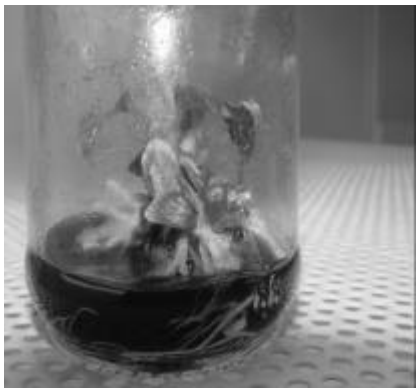

Fig. 2: Shoot elongation took place followed by rhizogenesis

Fig. 3: Hardened plantlet in a phytotron after transferring from *in vitro* conditions

## DISCUSSION

The process of *in vitro* plant regeneration via adventitious organogenesis in pepper (*Capsicum annuum* L.) using different sources of vegetative explants such as cotyledons, hypocotyls, roots and shoot tips has been widely discussed in literature (Ashrafuzzaman *et al*., 2009). The aim of the present
experiment was to study the effect of different cytokinins and auxins on shoot proliferation from four different explant sources of *Capsicum annuum* L.

Among different combinations of the regulators, a high concentration of cytokinin (BAP 6 mg $L^{-1}$) plus a low concentration of auxin (IBA 1 mg $L^{-1}$) was proven to be the best medium for direct organization of cotyledon explants. This was in accordance with other studies in which regenerated plants were obtained from cotyledons or hypocotyls at high levels of cytokinins (Hussain *et al*., 1999; Dabauza and Peña, 2001; Joshi and Kothari, 2007; Mezghani *et al*., 2007; Ashrafuzzaman *et al*., 2009; Guadalupe *et al*., 2009). In these studies, it was shown that regeneration only took place using high concentrations of BAP and low concentrations of auxins which both have important effects on direct organogenesis of the explants. Joshi and Kothari (2007) also showed that shoot buds of cotyledon explants were induced on medium supplemented with a high concentration of cytokinin and a relatively high concentration of auxin.

Favourable morphogenetic responses observed in cotyledons may be because they were obtained from young tissues, which have a higher capacity to respond to *in vitro* culture than explants from older material. This suggestion was confirmed by other reports in which young hypocotyl explants were successfully used for regeneration of *Capsicum annuum* (Andrzej *et al*., 2002; Ashrafuzzaman *et al*., 2009).

In present study, the proper medium for shoot elongation of the Iranian pepper was shown to be MS basal medium supplemented with plant growth regulators, BAP 1 and IBA 0.5 mg $L^{-1}$. Rhizogenesis of the elongated shoot took place again at the same medium as the one during the elongation period. The result was in accordance with those reported in some studies (Dabauza and Peña, 2001; Peddaboina *et al*., 2006; Guadalupe *et al*., 2009). Others found MS with $GA_3$ or $AgNO_3$ to be the best elongation medium (Mezghani *et al*., 2007; Ashrafuzzaman *et al*., 2009) and MS plus NAA or IBA to be the best medium for rooting of the elongated shoot (Hussain *et al*., 1999; Dabauza and Peña, 2001; Ashrafuzzaman *et al*., 2009) in sequential experiments.

The *in vitro* established plantlets were hardened in a phytotron with a survival rate of 70-90% and were then transplanted in glasshouse.

Overall findings of the present study are significant in obtaining the maximum regeneration with proper concentrations of growth regulator. In conclusion, we have developed a promising method for an efficient regeneration from cotyledon explants of *Capsicum annuum* L. using BAP and IBA. The protocol could be useful for large scale production of single genotypes like this cultivar and provides a possible system towards genetic improvement of the crop.

Organographic processes (or organ development) in plants include division, growth and differentiation of cells. These phases are genetically established and regulated by a number of exogenous and endogenous factors. Plant cells have a capacity to divide, grow and differentiate even when separated from an organism and growing on nutritional media. Therefore plant cells show totipotency including the ability to undertake and repeat the whole embryogenic development plan. In this work we trace the initial development of the regenerated bud-like structures on explants during *in vitro* cultures using basic histological staining method. These techniques produce important characterization of direct organogenesis in cultures of *Capsicum annuum* L.

Present experiment also indicated that the explants of the cotyledon include shoot buds of different stages of development. As seen in Fig. 4, after three days of culturing only parenchyma cells were observed, whereas a mature apical meristem was observed after 8 days (Fig. 5). The complete meristem with primordial leaves could be observed after 10 days (Fig. 6).

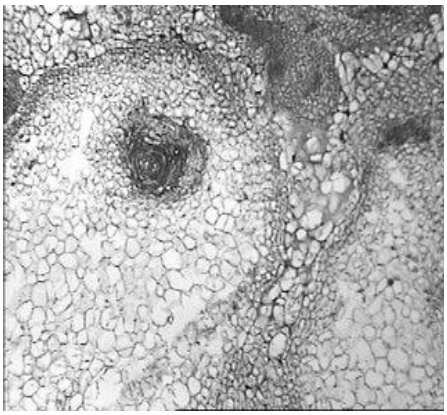

Fig. 4: Section of cotyledon parenchymatic cells after 3 days of culture. 30 μm

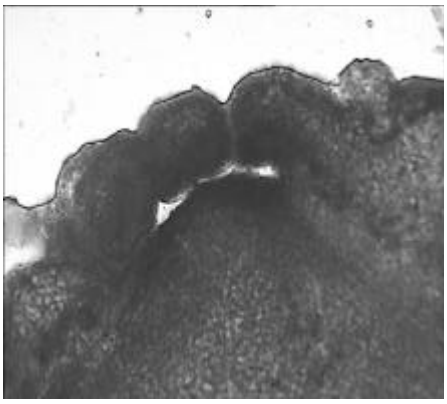

Fig. 5: Section of the embryo originated from the cotyledon after 8 days of culture. The development of the mature meristem was observed. 30 μm

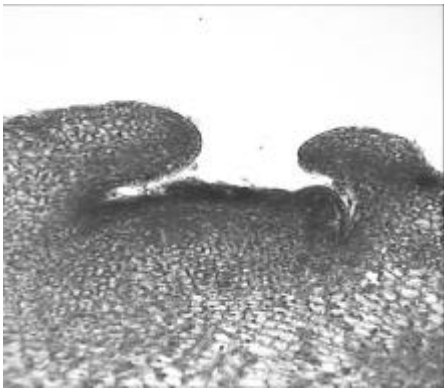

Fig. 6: Section of the embryo originated from the cotyledon after 10 days of culture. Meristem development with leaf primordial was observed on inside leaf tissue. 30 μm

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