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Original Contribution

THE EFFECT OF DIFFERENT CYTOKENINS IN PROPAGATION OF *CAPSICUM ANNUUM* L. BY *IN VITRO* NODAL CUTTING

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ABSTRACT

The application of modern biotechnology for improvement of pepper productivity requires an efficient *in vitro* plant regeneration protocol. In this study an experiment was carried out to examine the effects of different combinations of plant growth regulators on the *in vitro* micro propagation of *Capsicum annuum* L. thus optimizing the protocol for *in vitro* micro propagation involving culturing of nodal segments in MS media. Among the different concentrations of cytokinins BAP, KIN, Zea and TDZ tested. The best response was observed on medium containing 2 mg/l BAP and 0.5 mg/l IBA, especially for number of buds per explant and percentage of rooting. The elongated shoots were rooted on medium containing IBA (0.5 mg/l). Plantlets were transplanted to soil and acclimatized in the greenhouse showing normal development and growing to maturity bearing normal fruits with seeds.

Key words: *Capsicum annuum* L.; *In vitro* micro propagation; Nodal cutting; Plant growth regulators.

INTRODUCTION

Peppers are among the most important vegetable crops in the world, Chilies are the fruits of plants from the genus *Capsicum* of the nightshade family (Solanaceae). The genus *Capsicum* consists of about 25 wild and 5 domesticated species (1). Pepper, *Capsicum annuum* L., is considered as the economically most important species of the genus; the species includes both mild and pungent fruit types.

This method has some disadvantages, including short viability period, low rate of germination, and high risk of infection by various diseases. Pepper is sensitive to many pathogens containing fungi, bacteria, viruses and nematodes and also to extreme climatic conditions. Especially temperature extremes can limit its production. The application of modern biotechnology to enhance the productivity of pepper requires an efficient in vitro plant mass production protocol. In order to improve propagation of the commercial cultivars of this species and to meet the increasing demand for the crops, more reliable propagation approaches are needed for mass multiplication. Tissue culture aspects of pepper have been well studied (2, 3, 4, 5, and 6). Several tissue culture techniques to micropropagate pepper have been reported using different explants, including shoot tip (7) hypocotyl, leaf, stem, cotyledon, root, shoot tip and embryo (8) or applying induced somatic embryogenesis. However, many of these studies did not obtain satisfactory results with respect to the shoot number because the regeneration in this species is severely limited by formation of ill-defined buds or shoot-like structures which generally do not develop into normal shoots. Therefore, it is essential to design an effective protocol for in vitro micropropagation of pepper and for subsequent multiplication of the plantlets in the greenhouse.

Therefore, in the study, an attempt has been made to develop a simple, efficient in vitro propagation protocols for clonal propagation of the most popular cultivar of pepper plants by using Nodal segment from in vitro-propagation plants.

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In this present study, nodal cuttings of an endemic Iranian cultivar of *Capsicum annuum* L. were tested to establish in vitro micro propagation of the plant. The present study was also undertaken to show the relative efficiency of different combinations of plant growth regulators for in vitro regeneration from nodal explants and to select the best combination for maximal shoot initiation and elongation and establishment of complete plantlets.

MATERIAL AND METHODS

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

Seed sterilization and cultivation

The seeds were washed with tap water for 5 - 10 minutes to remove surface contamination and then sterilized by immersing in 70% ethanol for 1 minute with vigorous shaking followed by 20 minutes 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. For germinating, the seeds were cultured in a mug on 50 ml of standard MS medium (9) containing 3% (m/v) sucrose and 0.6% (m/v) agar. Cultures were incubated in a growth chamber at temperature of 24 °C, a 16 h photoperiod provided and with a light intensity of 2000 lux provided by white fluorescent lamps. After four weeks, the germinated seeds had produced young seedlings with 5-9 leaves. Nodal segments (with one node) derived from these aseptic seedlings were used as explants.

Nodal cutting and culture

Nodal segments (0.5 - 1 cm length) were cultured on MS medium supplemented with BAP, KIN, Zea or TDZ with three concentrations (0, 1.0, 2.0 mg/l) combined with IBA or NAA. Three concentrations (0, 0.2, 0.5 mg/l) of IBA or NAA were applied to enhance shoot formation. Sterilization was performed by autoclaving at 121 ºC for 20 min. pH was adjusted at 5.8 before adding 0.6% (w/v) agar and 0.4% (w/v) activated charcoal. Five explants (nodal segments) were aseptically cultured in a mug containing 50 ml of the induction medium. The mugs were covered and sealed with household plastic foil for a period of 5 weeks and then transferred to the same conditions as mentioned above.

Hardening and transfer

After 5 weeks, the rooted plantlets were acclimatized and transferred to pots containing sterilized peat mass and vermiculite (3:1 ratio). The pots were covered with a clear beaker with a few holes and were frequently watered to maintain a high humidity and kept in a phytotron for 10 days for hardening. Hardened plantlets were transferred to a greenhouse set at a day temperature of 21 ºC, a night temperature of 19 ºC, 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.

Data collection

Five weeks after transplanting, growth parameters including the number of branches, the number of leaves, the number of roots, the stem length, the average length of internodes, the average root length, and the percentage of rooting 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 (5 or 1%) based on the F-test, the Duncan's Multiple Range Test ($P \leq 0.05$) was used as a method to determine which treatments were statistically different from other treatments.

RESULT

In a preliminary experiment, MS medium with different concentrations of growth regulators the improved regeneration parameters of pepper obtained through adding AC to the media containing diverse kinds of plant hormones are shown in **(Fig. 3 and 4).**

Fig. 1. Callus induction in pre-existing buds of pepper after 2 weeks of culture. Note the extensive callus formation at the base of explants and the non-extended roots.

Fig. 2. Seedling of pepper with expanded buds and extended roots after 30 days of regenerating in medium containing activated charcoal plus plant growth regulators.

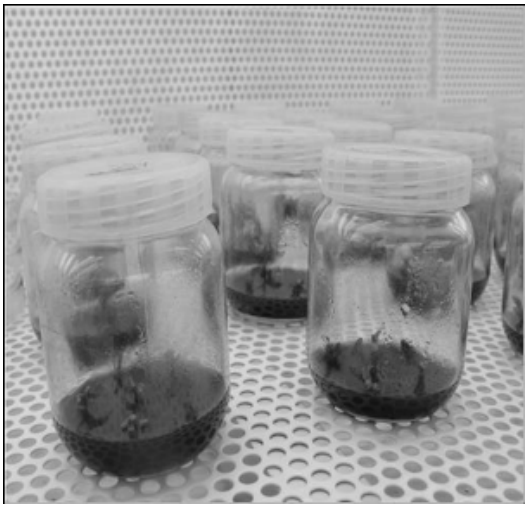
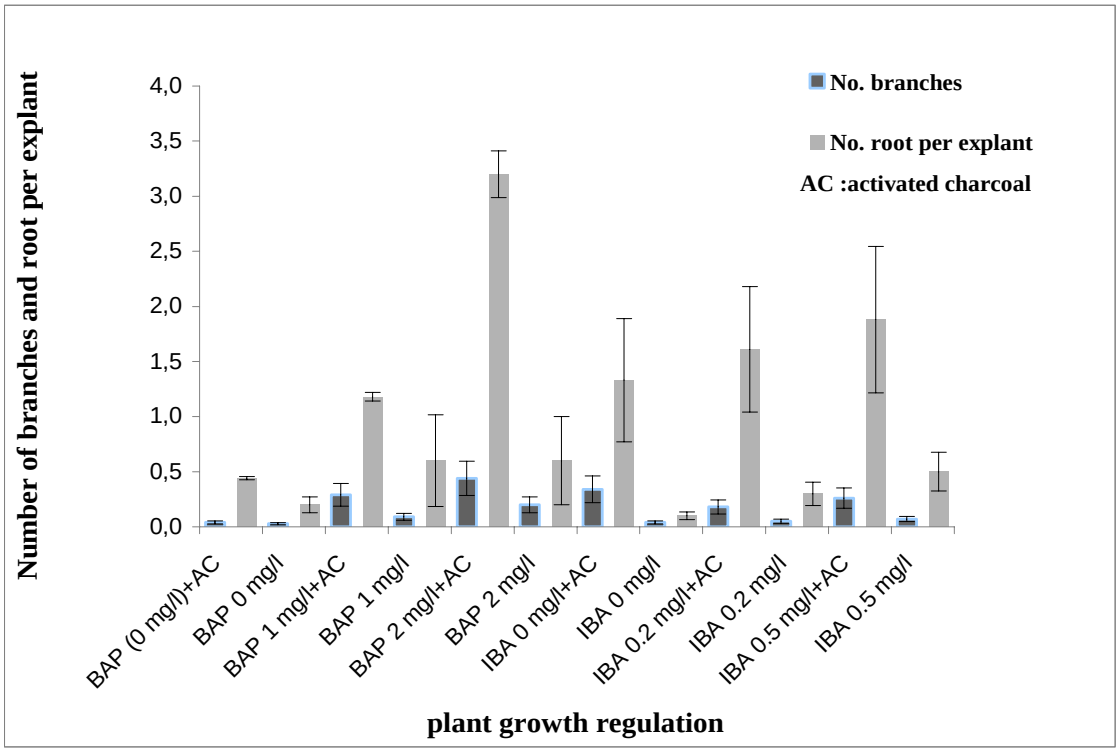


Fig. 3. Nodal segments of pepper taken from aseptic seedlings as explants on medium containing activated charcoal in combination with growth regulators.



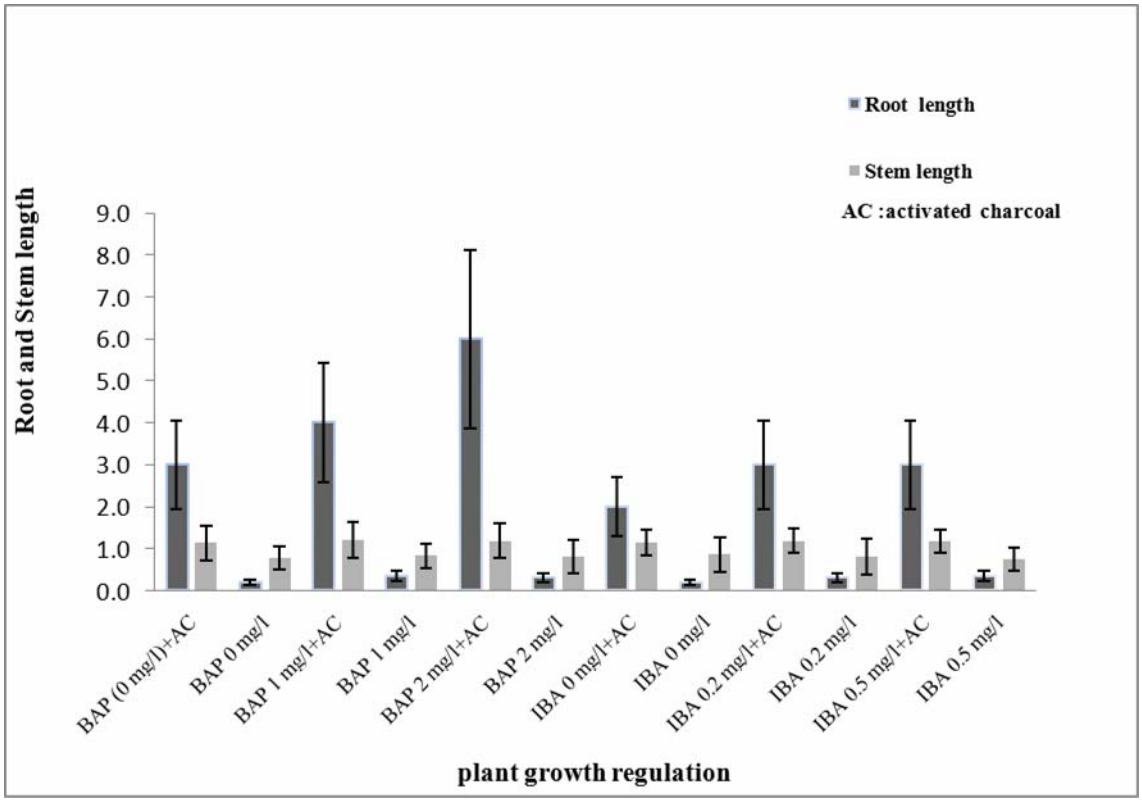


Fig. 4. Growth parameters of pepper in different kinds of regeneration media.

To optimize the media for regeneration of *C. annum*, different concentrations of the cytokinins BAP, KIN, Zea or TDZ alone or in combination with the auxins NAA or IBA were investigated. About 2-4 multiple shoot buds developed from explants derived from 2-3 old week seedlings germinated *in vitro* on the shoot bud induction media. Different concentrations and combinations of growth regulators showed significantly different

responses in terms of number of branches, leaves and roots per explant, the length of the stem, internodes and roots, and the percentage of rooting **(Tables 1 and 4)**. After 5 weeks of culture the maximum number of buds was obtained in MS medium supplemented with BAP with no auxin. The best concentration of BAP for bud induction was 2 mg/l, a treatment which produced 0.26 branches per explant **(Table 2)**. Media containing KIN, Zea or TDZ alone or in combinations with other growth regulators had the least effectiveness in inducing regeneration, as were especially illustrated by the number of branches per explant **(Table 4)**. The maximum number of roots per explant was obtained in MS combined with BAP (2 mg/l) without auxin **(Table. 2 and 4)**. However, the overall percentage of rooting was the highest in the medium containing BAP (2 mg/l) and IBA (0.5 mg/l) with that treatment approximately all explants properly rooted.

Table 1. *Mean sum of squares of the effects of BAP, KIN, Zea, TDZ, IBA and NAA and their two-way interactions on the number of branches, leaves and roots per explant, stem length, internode length, root length and the percentage of rooting of explants.*

Growth regulator	Number of branches	Number of leaves	Number of roots	Stem length (cm)	Internode length (cm)	Root length (cm)	Rooting (%)
BAP	0.02^{**}	123.35^{**}	74.00^{**}	32.12^{**}	0.22^{**}	465.00^{**}	33135.55^{**}
KIN	0.01^{ns}	15.02^{**}	1.91^{ns}	3.35^{**}	0.06^{*}	36.36^{**}	1382.96^{**}
Zea	0.01^{ns}	12.02^{*}	0.91^{ns}	2.34^{**}	0.04^{*}	18.26^{*}	1270.86^{**}
TDZ	0.02^{**}	100.00^{**}	67.00^{**}	22.10^{**}	0.20^{**}	324.02^{**}	27035.55^{**}
NAA	0.01^{ns}	2.59^{ns}	0.93^{ns}	0.22^{ns}	0.20^{ns}	1.68^{ns}	9.01^{ns}
IBA	0.01^{*}	9.68^{*}	0.49^{ns}	1.48^{**}	0.15^{**}	90.95^{**}	242.22^{ns}
BAP×NAA	0.00^{ns}	7.31^{*}	5.26^{ns}	0.13^{ns}	0.17^{**}	36.98^{**}	457.78^{ns}
BAP×IBA	0.02^{ns}	7.31^{*}	5.26^{ns}	1.13^{**}	0.17^{**}	36.98^{**}	457.77^{ns}
KIN×NAA	0.00^{ns}	3.96^{ns}	1.42^{ns}	2.13^{ns}	0.26^{ns}	1.92^{ns}	123.21^{ns}
KIN×IBA	0.00^{ns}	1.75^{ns}	0.94^{ns}	1.28^{ns}	0.02^{ns}	1.97^{ns}	136.3^{ns}
Zea ×NAA	0.00^{ns}	1.96^{ns}	0.42^{ns}	0.23^{ns}	0.16^{ns}	0.92^{ns}	100.21^{ns}
Zea ×IBA	0.00^{ns}	1.60^{ns}	0.54^{ns}	1.18^{ns}	0.09^{ns}	0.80^{ns}	96.3^{ns}
TDZ ×NAA	0.00^{ns}	6.41^{*}	4.12^{ns}	1.10^{ns}	0.19^{**}	26.78^{**}	227.00^{ns}
TDZ ×IBA	0.01^{ns}	6.40^{*}	4.20^{ns}	0.87^{*}	0.18^{**}	25.70^{**}	380.27^{ns}

Ns: Non significant ($p>0.05$), * Significant at $p\leq0.05$, ** Significant at $p\leq0.01$.

Table 2. *Effects of growth regulators BAP, IBA and NAA on the number of branches, leaves and roots per explant, stem length, internode length, root length and percentage rooting of explants.*

Treatment	Number of branches	Number of leaves	Number of roots	Stem length (cm)	Internode length (cm)	Root length (cm)	Rooting (%)
BAP (0 mg/l)	0.00 d	3.82 d	2.17 b	1.01 b	0.22 b	1.57 d	14.4 d
BAP (1 mg/l)	0.10 b	5.13 b	1.93 c	1.09 b	0.27 b	2.57 b	14.2 d
BAP (2 mg/l)	0.26 a	7.11 a	4.26 a	2.51 a	0.36 a	7.57 a	61.3 a
IBA (0.2 mg/l)	0.05 c	5.66 b	2.71 b	1.46 b	0.23 b	4.11 c	28.4 c
IBA (0.5 mg/l)	0.15 b	5.57 b	1.91 c	1.85 b	0.27 b	5.22 b	32.7 b
NAA (0.2 mg/l)	0.15 b	5.66 b	2.71 b	1.46 b	0.23 b	4.11 c	28.4 c
NAA (0.5 mg/l)	0.05 c	4.57 c	2.91 b	1.74 b	0.20 b	4.20 c	20.7 c

Means followed by the same letters within columns are not significantly different at the 5% level.

Table 3. *Effects of different concentrations and combinations of auxins (IBA and NAA) on the number of branches, roots per explants, root length and the percentage of rooting of explants.*

Growth regulators (mg/l)		Number of branches	Number of roots	Root length (cm)	Rooting (%)
IBA	**NAA**				
0	-	0.25 a	-	-	-
0.2	-	-	1.61 a	-	84.4 a
0.5	-	-	-	6.22 a	-
-	**0**	0.10 a	-	-	-
-	**0.2**	-	-	-	48.5 a
-	**0.5**	-	2.71 a	4.02 a	-

The values with letter a belonged to the best treatment.

Table 4. *Effects of different concentration growth regulators on the Number of roots per explants, root length* ***(cm)*** *and the percentage of rooting of explants.*

BAP (mg/l)	**KIN (mg/l)**	**Zea (mg/l)**	**TDZ (mg/l)**	**IBA (mg/l)**	**NAA (mg/l)**	**Number of roots**	**Root length (cm)**	**Rooting (%)**
1	-	-	-	0.2	-	1.61 c	3.40 c	48.9 b
2	-	-	-	0.5	-	2.70 a	7.57 a	61.3 a
1	-	-	-	-	0.2	2.17 ab	1.57 e	14.2 e
2	-	-	-	-	0.5	2.71 a	2.03 d	14.4 e
-	1	-	-	0.2	-	2.11 ab	3.11 c	28.4 d
-	2	-	-	0.5	-	1.72 c	3.22 c	32.7 c
-	1	-	-	-	0.2	1.16c	2.40 d	18.9 e
-	2	-	-	-	0.5	2.41 a	2.61 d	18.4 e
-	-	1	-	0.2	-	1.91 b	2.22 d	22.7 d
-	-	2	-	0.5	-	1.71 c	2.31 d	20.9 d
-	-	1	-	-	0.2	2.11 ab	1.64 e	11.3 e
-	-	2	-	-	0.5	2.00 ab	1.02 e	19.3 d
-	-	-	1	0.2	-	1.41 c	3.40 c	45.00 b
-	-	-	2	0.5	-	1.06 c	4.47 b	52.3 b
-	-	-	1	-	0.2	2.21 ab	1.14 e	28.02 d
-	-	-	2	-	0.5	2.07 ab	1.03 e	24.00 d
control	-	-	-	-	-	1.00c	2.40d	10.00 e

Means followed by the same letters within columns are not significantly different at the 5% level.

Following elongation, the non-rooted regenerated shoots (about 1.5 cm long) were separated and transferred to MS medium supplemented with IBA (0.5 mg/l) or NAA (0.5 mg/l) for rhizogenesis. IBA had a higher potential with respect to the induction of roots in this cultivar than NAA **(Table 3)**. The best result (more than 84% of the explants transferred showed root induction) was reached when the shoots were transferred to a medium containing 0.5 mg/l IBA. Such shoots rooted properly after 3 weeks of subculturing. Regenerated plantlets were used repeatedly as new materials for the next cycles of regeneration. Rapid multiplication of the plantlets was achieved within a short period of time. NAA produced stocky roots with fine root hairs, while the roots induced in media containing IBA were thin and long with many branches and root hairs **(Table 3)**.

The proliferated plants showed 70-90% survival during hardening and acclimatization **(Fig 5)**. There were no observable variations between the parent plants and *in vitro* propagated plants. The transplanted plantlets established well in a glasshouse **(Fig 6)**.

Fig. 5. Hardened plantlet of pepper in a phytotron after transferring from *in vitro* conditions.

Fig. 6. Pepper whole plants planted in a greenhouse.

DISCUSSION

The literature on regeneration of *Capsicum* and the role of plant growth regulators therein is diverse (10, 3, 8, 11, 12, 7, 13, and 14). These studies used different species, varieties and explants and a vast range of *in vitro* regeneration media and/or strategies. In this experiment was conducted to test the effect of various concentrations of different cytokinins; BA, Kin, TDZ,and zeatin on shoot proliferation in shoot explants of *Capsicum annuum* L. (Table 1) .The explants were showed different in the shoot proliferation response. The regeneration of *Capsicum annum* L. from nodal segments has been considered as a relative simple method, which could be potentially applied for mass propagation of the species.

Our preliminary experiment showed that adding activated charcoal (AC) in regeneration media enhanced the regeneration of explants with properly extended buds considerably (Table 4). In addition, AC induced roots with

good size. Nodal segments derived from the aseptic seedlings were used as explants and were put on a medium containing activated charcoal in combination with growth regulators. Removal of AC from the media led to proliferation of pre-existing buds with much callus induction at the base of explants after 2 weeks of culture. The positive effects of AC probably resulted from its property to bind and to absorb phenolics and other agents which could inhibit regeneration of the explants. This was in accordance with the results from studies of other plant species (15, 16). To the best of our knowledge our report is the first on the positive effect of AC on regeneration of the *Capsicum annum* L.

The best treatment of cytokinins producing the highest numbers of branches, leaves, and roots per explant was BAP 2 mg/l without auxin. The importance of BAP for regeneration of different cultivars of *Capsicum* has been emphasized by (7) who showed that MS medium supplemented with 10 mg/l BAP was effective for shoot multiplication in shoot-tip explants of *Capsicum annuum* L. cv. G4. (1) showed that MS medium supplemented with 5 mg/l BAP was effective for shoot multiplication in shoot-tip explants *Capsicum annuum* L. cv. Morok Amuba. In our study, the concentration of BAP higher than 2 mg/l was not effective for regeneration of pepper. Another cytokinin, KIN, alone or in combination with auxins was not as effective as BAP was for the regeneration of pepper. The others experiment that showed the maximum number of shoot proliferation amply demonstrates the high cytokinin activity of TDZ, as reported for several other species (17) also the others to get this result Zea proved to be the most effective for multiple shoot bud induction followed by BAP and Kin .The effectiveness of Zea alone or in combination with IAA (10), but in this experiment the minimum number of shoots was regenerated in media containing KIN, TDZ, Zea alone or in combination with IBA or NAA the other researchers that showed similar this result , This was in accordance with previously reports (8, 10, and 2).

NAA had a high potential in inducing rhizogenesis of the shoots (Table 3), however, in our experiment IBA was more effective than NAA with respect to rooting of the regenerated shoots (6) .The shoot buds proliferating from both shoot tips and axillary shoot explants rooted easily in medium supplemented with IBA. The axillary shoots further produced multiple shoot buds when cultured in bud induction medium. 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 minimum concentrations of growth regulator. In conclusion, we have developed a promising method for an efficient regeneration from nodal explants of *Capsicum annuum* L. using BAP and IBA. The protocol could be useful for large scale production of single genotypes and provides a possible system towards genetic improvement of the crop.

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Abbreviations: BAP: 6-benzylaminopurine; KIN: kinetin; NAA: α-naphthalene acetic acid; IBA: indole-3-butyric acid; Zea: Zeatin; TDZ: Thidiazuron; AC: activated Charcoal

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