Synergistic effect of silver nitrate and coconut water on shoot differentiation and plant regeneration from cultured cotyledons of *Capsicum annuum* L.

JB Mythili*, PR Rajeev, G Vinay & A Nayeem
Division of Biotechnology, Indian Institute of Horticultural Research, Hessaraghatta, Bangalore-560 089, Karnataka, India

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Chili pepper (*Capsicum annuum* L.) ranks among the most important vegetable crop belonging to the family Solanaceae that is consumed both as vegetable and spice throughout the world. *C. annuum*, as crop, in order to meet the target yield, demands improved variety that could overcome environmental challenges *viz.*, biotic and abiotic stress. Cultivar improvement essentially requires an efficient *in vitro* regeneration protocol. In the present study, we investigated the influence of silver nitrate (*AgNO₃*) and coconut water, individually as well in combination, on *in vitro* shoot elongation and plant regeneration from cotyledon explants of *C. annuum cv G-4*. Shoot buds were induced on shoot bud induction medium supplemented with either 44.38 µM 6-benzylaminopurine (BAP) or 9.0 µM thidiazuron (TDZ) along with 5.77 µM gibberellic acid (GA₃) and 14.7 µM phenyl acetic acid (PAA). Elongation of shoot buds was obtained on elongation medium containing 8.87 µM BA or 0.45 µM TDZ, 5.77 µM GA₃ and 14.7 µM PAA followed by rooting in 9.8 µM indole-3-butryic acid (IBA). All the media were supplemented with 30 µM *AgNO₃* and/or coconut water (10% *v/v*). The presence of coconut water in the elongation media enhanced the regeneration of well developed shoots from differentiating explants on TDZ media while *AgNO₃* resulted in enhanced production of rooted shoots with greater influence on emerging shoots from BAP media upon transfer to rooting media. There was synergistic response with further enhancement of elongated shoots/rooted shoots on the combined use of coconut water and *AgNO₃*. The elongation media produced significantly higher total shoots when *AgNO₃* was used synergistically with coconut water (59.0%) as against *AgNO₃* alone (38.0%). While in rooting media, there was significantly higher production of elongated rooted shoots when coconut water was used synergistically with *AgNO₃* (47.2%) as against the coconut water alone (14.4%).

**Keywords**: *AgNO₃*, Benzylaminopurine, Chilli pepper, Explants, Gibberellic acid, Indole-3-butryic acid, Phenyl acetic acid, Shoot buds, Thidiazuron

Chilli pepper, *Capsicum annuum* L. (Solanaceae), is an economically important crop known throughout the world as spice, and for its medicinal applications. The commercial importance of chilli pepper, has led to considerable progress in crop improvement through conventional breeding programmes. However, the development of plant biotechnology based cultivar improvement approaches have been protracted in chilli pepper for want of efficient *in vitro* regeneration protocols. This is because unlike other members of solanaceae for *e.g.* brinjal¹, *Capsicum* has been categorized as one of the most recalcitrant plant species for *in vitro* manipulations². Generally, in *in vitro* plant regeneration, there are reports of successful shoot regeneration from different explants through media manipulations³⁷. It is already established that the major bottleneck in the *in vitro* regeneration of *Capsicum* tissue cultures is the formation of ill-defined buds or shoot like structures either resisting elongation or producing rosettes of distorted leaves which generally do not produce normal shoots³⁹. Some of the strategies to overcome the difficulties in shoot-bud elongation include incorporation of the plant steroid lactone, 24-epibrassinolide⁸, silver nitrate¹⁰, phenyl acetic acid¹¹, higher copper levels¹² or regulating ethylene production or action¹³. However, inconsistency of these protocols with different genotypes and low reproducibility between laboratories suggest that efficient *in vitro* regeneration from seedling explants still remains a challenge³⁴.

Many plant species and varieties do not respond to classical approach of using MS¹⁴ media demonstrating that alterations in hormonal ratios cannot be the sole mechanism controlling *in vitro* developmental processes¹⁵. For such a difficult system, it is suggested to develop cultivar specific media formulations and incorporation of natural compounds such as coconut water may be a good alternative¹⁶. Such types of
complex addenda are known to possess mixed array of compounds and have been applied in tissue culture media successfully in the absence of desired results after using defined plant tissue culture media. In view of this, it is proposed in the present study, to supplement the tissue culture media having plant hormones with coconut water alone or in combination with AgNO₃ (which has helped in shoot elongation) to improve the efficiency of in vitro regeneration from cotyledon explants of chilli (Capsicum annum L.) cv G-4.

Materials and Methods

In vitro culture and regeneration

Seeds of chilli cv G-4 were decontaminated using sodium hypochlorite for 10 min, followed by 4-5 washings in sterile distilled water. MS medium with 3% (w/v) sucrose and solidified with 0.25% (w/v) phytagel, pH adjusted to 5.8 before autoclaving at 121°C and 1.2–1.3 kg cm⁻² pressure for 20 min was prepared for germination of seedlings. Five seeds were kept in a single test tube (10 mL medium in each). Cotyledons were excised aseptically from 10-12 day old seedlings producing two explants per seedling. Based on our earlier study, the media combinations for shoot induction, elongation and rooting were designed. The explants were placed in shoot induction media, MS media with phytohormones 9.0 µM TDZ or 44.48 µM BA; 5.77 µM GA₃ and 14.7 µM PAA. After the induction of adventitious shoot buds, the shoot buds arising from the cotyledons were excised and cultured on to elongation media comprising reduced levels of cytokinin 0.45 µM TDZ or 8.87 µM BAP along with same concentration of GA₃ and PAA. The regenerated elongated shoots were subcultured to 9.8 µM IBA containing rooting media. All the media were supplemented with or without 30 µM AgNO₃ and/or 10% coconut water (v/v). Media without any supplements served as control. The cultures were incubated at 25±1°C and RH (70%) under 16 h photoperiod (30-40 µE m⁻² s⁻¹) provided by white fluorescent tubes.

Experimental design and statistical analysis

There were 10 bottles per treatment with 6-8 explants per bottle and 3 bottles representing one replicate. Experiments were repeated at least thrice and the data was pooled before analysis. There were 3 replicates in different treatments in a completely randomized design (Table 1). The data indicated in the table are means of replicate values. The data in table were transformed using angular transformation and were subjected to analysis of variance (ANOVA). Comparison among treatment means were carried out using LSD values and are reported under “CD” at the end of each table.

Results and Discussion

Various stages of in vitro chilli regeneration from cotyledon explants viz., induction of shoot buds, shoot bud proliferation and elongation and rooted shoots are provided in Fig. 1 A-C. The cotyledon explants were cultured in different plant regulator combinations such as TDZ or BA with or without GA₃ and/or PAA. Explants gave rise to only callus in the absence of GA₃ in the shoot bud induction medium. Presence of GA₃ was found to be essential for inducing adventitious shoot buds. Earlier reports on chilli pepper regeneration have used BA and IAA for shoot bud induction. Subsequently, several reports have recorded the use of other cytokinins viz., kinetin, 2ip, zeatin and TDZ in combination with auxins such as IAA or NAA. However, GA₃ has been seldom used in shoot bud induction media while it was found to be critical for induction of adventitious shoot buds in cv G-4 used in the study. It may be attributed to the high genotypic effect on organogenic capacity reported by several workers. Gibberellic acid has been often used in combination with BAP as elongation promoter. Elongation of induced shoots has been considered to be the most limiting factor in in vitro chilli pepper plant regeneration.

In the present study, we incorporated AgNO₃ and or coconut water (CW) in all the steps of chilli pepper regeneration process. Influence of AgNO₃ varied with stage of regeneration, type of cytokinin used and also in combination with coconut water. AgNO₃ did not enhance shoot bud differentiation with least per cent explants (61.0) responding in AgNO₃ supplemented shoot induction media when averaged over the two cytokinin (TDZ or BA) media (Table 1) which was significantly lower than the media without any supplement. This is in contrast with Qin et al., who observed that explant differentiation could be dramatically increased with incorporation of AgNO₃ in the medium with maximum response obtained at 4 mg/l AgNO₃. They did not include AgNO₃ in the shoot elongation and rooting media. Similarly, Hyde and Phillips reported stimulation of bud enlargement, multiple shoot production and shoot elongation with 8.88 µM BA, 5.87 µM GA₃ and 5.89 µM AgNO₃ supplemented media. Compared to
AgNO₃, incorporation of coconut water significantly increased % explants (92.5) giving rise to shoot buds but did not vary significantly from the media without any supplement (90.0) Table 1. Thus, at the shoot bud differentiation stage, coconut water did not have any significant positive influence while AgNO₃ significantly reduced the response.

The ability to initiate shoot development from regenerated shoot buds in both the cytokinin media was favoured by addition of coconut water in
shoot elongation media resulting in 66.0% shoot regeneration while presence of AgNO₃ significantly reduced shoot initiation response (38.0%) compared to media without any supplement (51.0%). However, AgNO₃ acted synergistically with coconut water giving significantly higher % shoots regenerating (59.0%) than when it was used alone. Coconut water supplementation in elongation media also resulted in greater % (14) of upright shoots (>1.5 cm) from the total shoots generated when used alone, 17% upright shoots when used in combination with AgNO₃ and only 2% upright shoots when AgNO₃ was used alone (Table 1).

The induction of upright shoots was more pronounced in TDZ media as greater % upright shoots (15 & 25, Table 1) from the total shoots initiated were obtained when coconut water was used alone or in combination with AgNO₃, respectively compared to only exclusive AgNO₃ supplementation that resulted in merely 3.0 % upright shoots. In BAP containing elongation media, coconut water supplementation alone gave rise to 13% upright shoots while media with presence of AgNO₃ alone or in combination with coconut water was characterized by total absence of upright shoots (Table 1). Such potency of TDZ in inducing maximum number of adventitious shoots has been reported already and claimed that TDZ is better than BA or other cytokinins. Venkataiah et al. reported thidiazuron (TDZ)-mediated organogenesis in 10 pepper cultivars. A highly efficient procedure for shoot multiplication and plant regeneration of Capsicum was developed by Venkataiah et al. wherein various cytokinins were tested and found that TDZ regenerated maximum number (4.2–22.4) of shoots in all the Capsicum species tested.

The emerging/upright shoots were transferred to rooting media (IBA 2 mgl⁻¹ with AgNO₃ and/or coconut water) for further elongation and rooting. At this stage, there was enhanced response in regeneration of elongated rooted shoots in AgNO₃ supplemented rooting media with significantly higher % of elongated rooted shoots when AgNO₃ was used alone (26.2) or in combination with coconut water (47.2) while the coconut water supplemented media gave the least % of elongated rooted shoots (14.4). The effect of AgNO₃ in inducing elongated rooted shoots was pronounced in shoots regenerated from BAP media as compared to TDZ media giving rise to 33.3 and 19.1% elongated rooted shoots respectively when AgNO₃ was used alone (Table 1).

These results suggest that the effect of coconut water is more pronounced in the elongation stage while AgNO₃ influenced the rooting phase. Coconut water facilitated recovery of 21 and 28.8% upright shoots in elongation media when coconut water was used alone or in combination with AgNO₃, respectively while only 5.2% upright shoots were obtained in AgNO₃ supplemented media. On the other hand, the effect of AgNO₃ is delayed with greater expression in rooting media. There was 13.0x and 2.8x greater conversion of rooted shoots from upright elongated shoots in rooting media when AgNO₃ was used alone or in combination with coconut water, respectively as compared to use of coconut water alone in rooting media (Table 1). In fact, root initiation was observed as early as 102 days in rooting media supplemented with AgNO₃ followed by 120-130 days for media supplemented with AgNO₃ and coconut water and it took about 140 days in coconut water supplemented media. Silver ions in the form of AgNO₃, has played a major role in efficient root formation through regulation of ethylene action and/or production, and thus bring about its effect on shoot elongation. The production of ethylene in in vitro cultures can affect callus growth, shoot regeneration and somatic embryogenesis in vitro. Batista et al. suggest that the use of ethylene inhibitors and the addition of increasing concentrations of polyamines may be important tools for reducing ethylene levels and reducing in vitro recalcitrance of C. annuum.

The synergistic effect of coconut water and AgNO₃ was observed both in elongation as well as rooting media. In the elongation media, there was significantly higher production of total shoots when AgNO₃ was used synergistically with coconut water (59.0%) as against the use of AgNO₃ alone (38.0%). Synergy of coconut water and AgNO₃ also translated in greater recovery of upright shoots (17%) as against 2% upright shoots in AgNO₃ media. In rooting media there was significantly higher production of elongated rooted shoots when coconut water was used synergistically with AgNO₃ (47.2%) as against the use of coconut water alone (14.4%). In both elongation and rooting media, the synergistic response was significantly enhanced in TDZ media with greater % of elongated shoots (66.0) and elongated rooted shoots (51.7) as compared to 46 and 42.6% elongated shoots and elongated rooted shoots, respectively in BAP media. It was also observed that the combined
use of AgNO₃ and coconut water resulted in well elongated rooted shoots that reached the top of the culture bottle while in AgNO₃ or coconut water supplemented media, the elongated rooted shoots reached middle of the culture bottle and in some cases, rooting was delayed in coconut water supplemented media (Figs. 2-4). These results suggest that coconut water exerted its influence at the elongation stage while the effect of AgNO₃ was more pronounced at the rooting stage.

To the best of author’s knowledge, we have not come across any reports of influence of coconut water on chilli pepper regeneration although coconut milk was used in the very first report of Capsicum organogenesis by Gunay and Rao. Based on this report, either use of coconut water was not attempted or its incorporation in the media did not prove useful. Such observations are expected considering the huge genotypic influence on organogenic ability. The results of the present study report the positive influence of coconut water in in vitro regeneration. Coconut water in induction media resulted in highest % explant differentiation of shoot buds. It also exhibited synergistic influence with AgNO₃ during regeneration of upright shoots in elongation media. The synergism was more pronounced in TDZ containing elongation media. TDZ is emerging as a more potent cytokinin in Capsicum regeneration.

Coconut water, the colourless liquid endosperm contains a number of amino acids, organic acids, nucleic acids, vitamins, sugars, sugar alcohols, plant hormones (auxins, cytokinins) and other unidentifiable
compounds, none of which is solely responsible for growth promoting qualities. Unlike other complex nutrients such as malt or yeast extract, casein hydrolysate, coconut water has proved harder to replace by fully defined media. Many workers try to avoid using coconut water in their protocols due to the lack of reproducibility. However, adding coconut water provides a simple way to obtain satisfactory growth or morphogenesis without the need to work out a suitably defined medium\(^3\). In addition to the standard component of the media, in case of specific needs of a particular species or tissues, other components including organic nitrogen compounds, organic acids and a variety of complex natural extracts, can be important but optional. When completely defined media did not give desired results, employing coconut water (milk), other fluid endosperms, malt/yeast extract, tomato, carrot and orange juice have beneficial effects on \textit{in vitro} plant cell and tissue cultures\(^3\).

The exact conditions required to initiate and sustain plant cells in culture, or to regenerate intact plants from cultured cells, are different for each plant species. The empirical approach has shown that three factors, namely explant choice, medium composition, and control of the physical environment are important in successful cultures. When the completely defined plant culture media failed to give the desired results, employing natural substances have beneficial effects on \textit{in vitro} plant cell and tissue cultures\(^3\).

The present study on recalcitrant chilli pepper reiterates this fact, as coconut water, a complex addendum in combination with AgNO\(_3\) was successful in overcoming the major bottleneck of shoot elongation in \textit{in vitro} chilli pepper regeneration process and improving the efficiency of \textit{Capsicum} regeneration.

**References**


