ISSN:2319-2607 IISUniv.J.Sc.Tech. Vol.5(1),24-28 (2016)
RESEARCH ARTICLE

Micronutrient Optimization in Culture Media Improves Plant Regeneration in *Capsicum annuum* L.

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Abstract

Indian traditions have long been utilizing various spices for culinary and medicinal purposes. They have a wide range of biological functions and their synergistic effects are likely to protect the body against various ailments. Chilli is an economically important plant cultivated worldwide which is used both as a spice and vegetable. Chilli plants are prone to fungal and viral pathogens which account for heavy economic losses. Traditional breeding programs are of great value for increasing yield and productivity but biotechnological techniques involving plant tissue culture and recombinant DNA technologies could be powerful auxiliary tools to achieve this goal. *Capsicum* is recalcitrant with regard to its *in vitro* regeneration efficiency, which makes it difficult to apply recombinant DNA technologies via genetic transformation aimed at genetic improvement against pests and disease. Therefore, the effect of cobalt chloride on differentiation of shoot buds from cotyledon explants of *Capsicum annuum* L. was investigated with an aim to improve tissue culture response. Shoot buds were induced on MS medium supplemented with BAP (5 mg/l) + PAA (2 mg/l). Elongation of shoot buds was done on MS medium containing BAP (3 mg/l) + GA_3 (0.2 mg/l). Both shoot induction and elongation media were supplemented with different concentrations of $CoCl_2$ (0, 0.11*, 0.55, 1.1, 2.2, 3.3, 5.5µM). Incorporation of $CoCl_2$ in the media significantly improved the regeneration efficiency of *Capsicum* cultures.

Keywords: *Capsicum annuum,* Cobalt chloride, Micropropagation, MS media, Regeneration

Introduction

The diversity of forms, color, shapes, flavor and pungency makes *Capsicum* an important, virtually indispensable ingredient in the culinary art. It is used both as a spice and vegetable. Besides being an important food crop, it exhibits antimicrobial and pharmacological properties. The production potential of chillies does not often reach the achievable targets due to wide variety of abiotic and biotic factors. Chilli pepper plants show high level of heterogeneity in seed population, which acts as a limitation for propagation of agronomic traits and for commercial seed production. Conventional methods utilizing huge amounts of seeds result in wastage of the crop significantly. The propagation through seeds is additionally restricted by short span of viability and low germination rate of the seeds. A great menace to the productivity of *Capsicum* is its vulnerability to viral pathogens which causes severe damage to the standing crop. Traditional breeding techniques have been of substantial value for chilli pepper genetic improvement but increase in yield and productivity cannot be sustained indefinitely using the primary gene pool. So, biotechnological techniques involving plant tissue culture and recombinant DNA technologies could be powerful ancillary tools to accelerate this goal. These techniques are becoming functional aspect of classical breeding programs. Tissue culture based technologies provide a crucial adjunct not only to conventional breeding but also for the propagation and genetic improvement of plants. One of the most important steps in developing a reproducible protocol for successful propagation and *in vitro* storage is selecting the appropriate culture medium. Development of an suitable growth medium for a specific crop can be quite complex because the response to the culture medium is often genotype- dependent and the effects of mineral nutrition on morphogenesis are poorly understood (Ramage and Williams, 2002; Greenway *et al.,* 2012). Many slow growing or recalcitrant species and cultivars do not respond to the classical optimization approach of trying plant growth regulators (PGRs) or screening existing medium formulations such as Murashige and Skoog (MS)(Murashige and Skoog, 1962). Minerals play an important role in the regulation of both plant morphogenesis and growth (Ramage and Williams, 2002). Nutrient deficits are well studied and documented in field plants (Bennett, 1993),but have not been commonly studied *in vitro*. Preece (1995) suggested that plant growth on suboptimal nutrient media may be compensated for by

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higher PGR concentrations, and media with optimal nutrients may require lower PGR concentrations for healthy plant growth. To improve the growth of *in vitrocapsicum* shoots, the objective of this study was to determine the effects of micronutrient $CoCl_2$ on the induction and elongation of *capsicum* cultures and to determine the optimum nutritional requirement of the micronutrient.

Materials and Methods

Establishment of Aseptic Seedlings and Explant Preparation

Seeds of *Capsicum annuum*L. cv X-235 were first washed with 20% (v/v) Extran (Merck, India) followed by 3-4 washings with sterile distilled water. Seeds were then soaked in sterile distilled water for 2 days, kept on rotary shaker, and were maintained at temperature 26±1°C. These seeds were surface-sterilized with aqueous solution of 0.1% $HgCl_2$for 3 min and rinsed three times with sterile distilled water. MS medium having 2% (w/v) sucrose and solidied with 0.8% (w/v) agar (Qualigens, bacteriological grade), 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. Seven seeds were kept in a ask (100 ml 'Erlenmeyer' with 40 ml medium in each). All the cultures were incubated in growth chamber at a temperature of 26±1°C and 16 h photoperiod and light intensity of 25 mol/m^2/s provided by white uorescent tubes. Cotyledons explants were taken from 20 day old seedlings.

Culture Media

The cotyledons with their dorsal surface in contact with the medium were cultured on the MS medium augmented with BAP (5 mg/l) and PAA (2 mg/l) and sucrose 3% (w/v). This was considered as control induction medium having 0.11µM $CoCl_2$ already present as a micronutrient in MS basal medium composition .The levels of cobalt chloride were varied as (0, 0.11*, 0.55, 1.1, 2.2, 3.3, 5.5µM). Five asks (100 ml 'Erlenmeyer' with 40 ml medium in each) were prepared for each treatment having three explants per ask. Visual observations were made for 5 weeks. Percentage response was calculated by dividing the total number of responding explants by total number of explants inoculated. Shoots buds induced at the base of cotyledons were excised and cultured on normal shoot elongation medium EM1 (MS medium supplemented with 3 mg/l BAP and 0.2 mg/l GA_3 and $CoCl_2$ as present in normal MS medium) and on modied elongation medium EM2 (MS medium supplemented with 3 mg/l BAP and 0.2 mg/l GA_3 and varied levels of $CoCl_2$ 0, 0.11*, 0.55, 1.1, 2.2, 3.3, 5.5µM). Weekly observations were recorded for 4 weeks.

Rooting and Acclimatization

Elongated shoots 2 cm and more in length were excised and transferred to rooting medium consisting of full strength MS medium fortified with PAA (1 mg/l). Plantlets with well- developed shoot and root systems were cautiously taken out of the test tubes and washed with tap water to remove any trace of agar on the roots. These plantlets were transferred to earthen pots having garden soil and organic manure (1:1). Humidity was maintained initially by covering the pots with polythene bags. The experiment was repeated three times.

Results and Discussion

The experiment was conducted in two phases. In the first phase, a standard protocol was established to determine the most suitable hormonal combination for regeneration and then further studies were conducted with modied levels of cobalt chloride in the proposed medium.

Shoot Regeneration

Cotyledonary explants obtained from aseptically raised seedlings were cultured with their dorsal surface in contact with the medium. Differentiation of shoot-buds from the cut ends of the explant was observed on all combinations of BAP (3- 7 mg/l) and PAA (0.5-2 mg/l). 2-3 normal shoots were formed from the cut end of cotyledons on medium with BAP (3 mg/l) and PAA (0.5 mg/l). Increasing the levels of both BAP and PAA in the culture media improved the number shoot bud initiated per explant. Best response was obtained on medium supplemented with BAP (5 mg/l) in combination with PAA (2 mg/l)where an average of 8 normal, healthy shoot-buds was induced. Shoot-buds differentiated on induction medium were smaller in size and needed further elongation, so they were sectored into clumps of 2-4 and sub-cultured on different hormonal regimes for elongation of the smaller buds. Lower levels of BAP (0.5-1 mg/l) in combination with GA_3 (0.2-1 mg/l) were not effective, whereas, higher level of BAP (3 mg/l) and low level of GA_3 (0.2 mg/l) was found to be most effective for elongation of primary shoots and their proliferation as well.

Nutrient Manipulation Studies

The regeneration potential of plants is known to be inuenced by extrinsic supply of nutrients in the tissue culture media. Micronutrients are essential components

of several enzymes (Maksymiec, 1997) and act as secondary messengers that help in regulating and monitoring plant tissue growth (Niedz and Evens, 2007). Inorganic nutrients are major components of MS medium and hence offer to be the best variable to study their effect on the morphogenetic potential of the plant.In the present study, effect of $CoCl_2$on shoot morphogenesis in *capsicum* was studied.The micronutrient exerted a profound influence on differentiation of shoot-buds and their subsequent elongation in *Capsicum*. The shoot bud-forming capacity of cotyledonary explants cultured on different levels of the micronutrient was signicantly improved. The percentage of responding explants increased with the increasing concentrations of $CoCl_2$ (Table 1, 2; Fig.1a, b). The most prominent effects were shown by cultures initiated on 1.1 µM $CoCl_2$ which is 10 times the normal level of cobalt chloride in the normal induction medium. An average of 12 shoot-buds per explant was induced on this modified medium. Further increase, in the level of the nutrient was not beneficial for shoot bud formation. Thus, the increase in concentration of the nutrient exhibited a synchronized improvement in the regeneration response up to a threshold beyond which there was deterioration in the regeneration efciency. Elongation and further multiplication of shoot-buds was also influenced by nutrient manipulation. Shoot-buds when sub-cultured to elongation medium with elevated concentrations of $CoCl_2$ presented better results. Best response was observed when shoot-buds differentiated on 1.1 µM $CoCl_2$ concentrations (Fig. 2.a, b) were sub-cultured on similar level of the nutrient for subsequent elongation and were better than those sub-cultured on normal elongation medium with pre-determined level of the mineral in MS media. Well elongated plantlets were transferred to rooting medium consisted of MS medium supplemented with PAA (1mg/l). Thick long and healthy roots were induced on this medium. Well rooted plantlets were acclimatized and transferred to field conditions (Fig. 3 a, b).

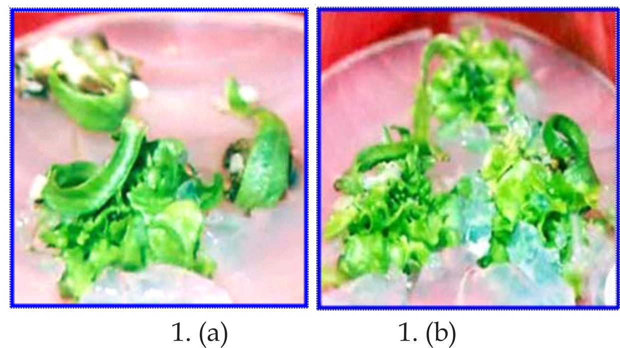
Table 1: Effect of Various Concentrations of $CoCl_2$ on Induction of Shoot Buds from Cotyledon Explants of *Capsicum annuum* L.

CoCl2(*µM*) in induction medium*	% Response	No. of shoot buds per explant Mean±S.D.
0	*55*	*3.6 ± 01.2*
0.11ᶜ	*72*	*7.1 ± 1.2*
0.55	*83*	*8.3 ± 1.1*
1.1	**94**	**12.3 ± 1.5**
2.2	*77*	*9.8 ± 1.3*
3.3	*77*	*9.2 ± 1.7*
5.5	*61*	*7.9 ± 1.6*

Table 2: Effect of Various Concentrations of $CoCl_2$ on Elongation of Shoot Buds of *Capsicum annuum* L.

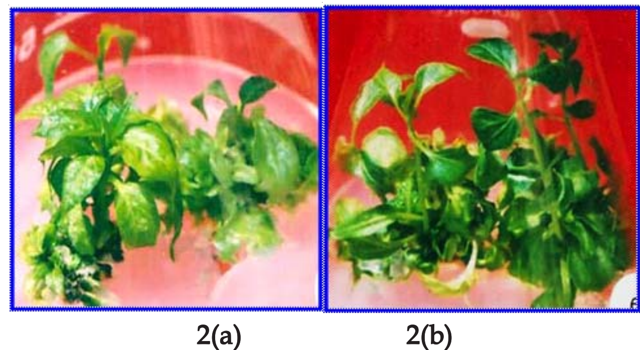
$CoCl_2$(*µM*) in induction medium*	$CoCl_2$(*µM*) in elongation medium**	No. of shoot buds per explant Mean±S.D.	No. of shoot buds elongated
	0	*8.2 ± 1.3*	3
0	*0.11ᶜ*	*9.8 ± 1.4*	3
0.11ᶜ	*0.11ᶜ*	*13.6 ± 1.5*	5
	0.11ᶜ	*14.2 ± 1.4*	5
0.55	*0.55*	*17.6 ± 1.6*	7
	0.11ᶜ	*16.4 ± 0.8*	6
1.1	**1.1**	**24.8 ± 1.3**	**11**
	0.11ᶜ	*15.1± 0.5*	6
2.2	*2.2*	*18.6 ± 0.5*	8
	0.11ᶜ	*12.4 ± 1.1*	4
3.3	*3.3*	*14.6 ± 1.5*	5
	0.11ᶜ	*11.8 ± 1.7*	4
5.5	*5.5*	*15.2 ± 0.8*	4

C = Control



1. (a) 1. (b)

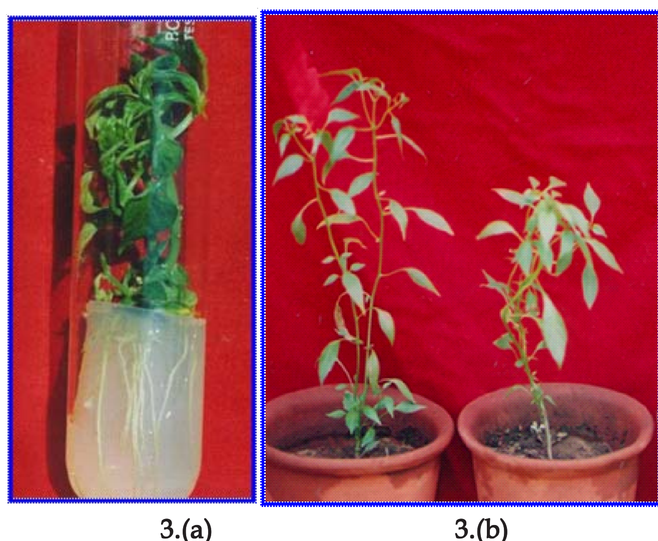
Fig. 1 : Effect of $CoCl_2$ on Shoot Bud Formation from Cotyledons of *Capsicum annuum* Cultured on MS Medium Supplemented with BAP (5 mg/l) + PAA (2 mg/l) (a.) *0.11 µM $CoCl_2$ (b.) 1.1 µM CoCl2



2(a) 2(b)

Fig. 2: Effect of $CoCl_2$ on Elongation of Shoot-Buds of *Capsicum annuum* Cultured on MS Medium Supplemented with BAP (3 mg/l) + GA3 (0.2 mg/l) (a.) *0.11 µM $CoCl_2$ (induced on similar medium) (b.) 1.1 µM $CoCl_2$ (induced on similar medium)

In accordance to the present study, Roustan *et al.*,(1989) also studied the effect of cobalt on the stimulation of embryogenesis in carrot. They attributed the stimulatory effect either to a direct influence of $CoCl_2$ on the embryo formation or to an inhibition of ethylene production. (Yang and Hoffman, 1984; Kintzios *et al.,* 2001) reported varying effects of cobalt concentration on somatic



3.(a) 3.(b)

Fig. 3. Rooting and Field Transfer of Regenerated Plants (a) Rooting Response of *in vitro* Regenerated Shoots of *Capsicum annuum* Cultured on MS Medium Supplemented with PAA (1 mg/l); (b) Field Transferred Plantlets

embryogenesis depending upon the concentration. Higher concentration of $CoCl_2$, both at induction and subculture promoted shoot bud formation in barley (Chauhan and Kothari, 2004). Signicant improvement in plant regeneration was also observed with the increase in levels of $CoCl_2$ in both *Paspalum* and *Eleusine* (Kothari-Chajer *et al.,* 2008).Optimization of micronutrientsMnSO_4, KI and $CoCl_2$in the MS basal culture medium resulted into enhanced *in vitro* plant regeneration, chlorophyll content and biomass in *Stevia rebaudiana* (Bert.) Bertoni(Jain *et al.,* 2012). Cobalt chloride has proved to be an important heavy metal for improvement of plant growth in *in vitro* conditions and at the same time has also shown positive response in *in vivo* conditions.Gad and Hassan, (2013) evaluated the effect of cobalt nutrition (0.0, 2.5, 5.0, 7.5 and 10.0 ppm) on sweet pepper growth and productivity. The results indicated that all cobalt concentrations significantly increased all growth and yield parameter compared with controlplants.In the present study, higher concentrations of $CoCl_2$ (10 times than MS) enhanced the induction of shoot-buds and their subsequent elongation in *Capsicum*. Contrary to this, Witte *et al.,* (2002) completely rejected the role of $CoCl_2$ in plant tissue culture. They reported that presence of $CoCl_2$ leads to accumulation of urea which is not favourable for plant growth. $CoCl_2$ has been reported to increase betalains production in *Beta vulgare* (Trejo-Tapia *et al.,* 2001) which inhibits morphogenesis in both rice and barley at concentrations higher than the optimum. A toxic effect of $CoCl_2$was also witnessed at levels higher than the optimum in *Capsicum*. Similar effect of $CoCl_2$ at high concentrations has been reported by Murashighe and Skoog, (1962). The results of the present study are also in accordance with those of Roustan *et al.,* (1989) where high level of $CoCl_2$ was found to affect cell survival. It triggered the formation of embryos with brown cotyledons and completely inhibited transformation of embryos to plantlets and thus exerted a toxic effect. The prototypes produced in this study clearly indicated that the components of MS medium require adjustment for the best growth of various genotypes and requires extensive evaluation of tissue culture medium to optimize the regeneration response.

References

Bennett, W.F. (ed.) (1993) Nutrient deficiencies and toxicities in crop plants. APS Press, Minneapolis, MN, 202 pp.

Chauhan, M., Kothari, S.L. (2004) Optimization of nutrient levels in the medium increases the efficiency of callus induction and plant regeneration in recalcitrant Indian Barley (*Hordeum vulgare* L.) *in vitro. In Vitro Cell Dev Biol Plant* **40**: 520-527.

Greenway, M.B., Phillips, I.C., Lloyd, M..N., Hubstenberger, J.F., Phillips, G.C. (2012) A nutrient medium for diverse applications and tissue growth of plant species in vitro. *In Vitro Cell Dev Biol Plant* **48**:403-410

Jain P., Kachhwaha S. and Kothari S.L. (2012) Optimization of micronutrients forthe improvement of *in vitro* plant regeneration of *Stevia rebaudiana* (Bert.) Bertoni. *Indian J Biotech* **11**: 486-490

Kintzios, S., Drossopoulos, J.B., and Lymperopoulos, C. (2001) Effect of vitamins and inorganic micronutrient on callus growth and somatic embryogenesis from leaves of chilli pepper. *Plant Cell Tiss Org Cult* **67**: 55-62.

Kothari-Chajer, A., Sharma, M., Kachhwaha, S., Kothari, S.L. (2008) Micronutrient optimization results into highly improved in vitro plant regeneration in kodo (*Paspalum scrobiculatum*L.) and nger (*Eleusine coracana* (L.) Gaertn.) millets. *Plant Cell Tiss Org Cult* **94**:105-112

Maksymiec, W. (1997) Effect of copper on cellular processes in higher plants. *Photosynthetica* **34**:321-342

Murashige, T., Skoog, F. (1962).A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* **15**: 473-497.

Gad, N., Hassan, N.M.K. (2013). Response of Growth and Yield of Sweet Pepper (*Capsicum annuum* L.) to Cobalt Nutrition. *World Appl Sci J* **2**: 760-765.

Niedz, R., Evens, T.J. (2007) Regulating plant tissue growth by mineral nutrition. *In Vitro Cell Dev Biol Plant* **43**:370-381

Preece, J,E, (1995). Can nutrient salts partially substitute for plant growth regulators? *Plant Tiss Cult Biotechnol* **1**: 26-37.

Ramage, C.M., Williams, R.R. (2002). Mineral nutrition and plant morphogenesis. *In Vitro Cell Dev Biol Plant* **38**: 116-124.

Roustan, J.P., Latche, A., Fallot, J. (1989) Stimulation of *Daucus carota* somatic embryogenesis by inhibitors of ethylene biosynthesis: cobalt and nickel. *Plant Cell Rep* **8**: 182-185.

Trejo-Tapia, G., Arias-Castro, C., Rodriguez-Mendiola, M. (2003) Influence of the culture medium constituents and inoculum size on the accumulation of blue pigment and cell growth of Lavandulaspica. *Plant Cell Tiss Org Cult* **72**: 7-12.

Witte, P.C., Tiller, S.A., Taylor, M.A., Davies, H.V. (2002) Addition of nickel to Murashige and Skoog in plant culture activates urease and may reduce metabolic stress. *Plant Cell Tissue Organ Cult* **68**:103-104

Yang, S.F., Hoffman, N.E. (1984) Ethylene biosynthesis and its regulation in higher plants. *Ann Rev Plant Physiol* **35**: 155-189.