International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 16-12-2021, Accepted: 01-01-2022, Published: 17-01-2022
Volume 7, Issue 1, 2022, Page No. 325-329

Silver nitrate: A potent anti-ethylene compound stimulates organogenesis in naga chilli (*C. chinense* Jacq.)

Geetashree Bora[1], Pratap Jyoti Handique[2]
[1] Department of Botany, Assam Don Bosco University, Tepesia, Sonapur, Assam, India
[2] Gauhati University, Guwahati, Assam, India

Abstract
Northeast India is considered to be one of the prominent centres of chilli pepper diversity. Naga chilli locally known as Bhot jolokia is extensively cultivated all over northeast India including Assam. This chilli possesses high commercial value owing to pungency factor called capsaicin. Despite its tremendous pharmaceutical properties, the germplasm is at risk owing to natural cross pollination and mutation thereby reducing its capsaicin content. This communication brings highlights a complete standardized *in vitro* micropropagation protocol in Naga Chilli (Bhot jolokia) from leaf explants under the influence of silver nitrate. *In vitro* propagation was carried out with leaf explants. MS medium was used as the basal medium supplemented with different combinations of plant growth hormones. Addition of Silver nitrate (AgNO3) to the culture medium along with phytohormones (BAP, GA3, 2,4-D and IBA) greatly improved proliferation in the selected *in vitro* plantlets. AgNO3 effectively induced prolific regeneration of multiple shoots. *In vitro* flower and fruit induction processes were found to be more productive in the treatment where AgNO3 was combined with GA3 in the culture medium. *In vitro* root initiation in Naga chilli was very responsive in MS medium supplemented with IBA Potting material fortified with vermicompost and vermiculite greatly improved the chances of acclimatization of *in vitro* raised healthy plantlets. Plant survival percentage was 86.65%.

Keywords: AgNO3, *In vitro* organogenesis, naga chilli, phytohormones

Introduction

North east India is known to be one of the hotspots of pepper diversity. The genus Capsicum of the Solanaceae family consists of approximately 25 wild and 5 domesticated species [1] *C. annuum* L., *C. chinense* Jacq., *C. frutescens* L., *C. baccatum* L., and *C. pubescens* are the top five cultivated species and the first three are cultivated extensively in India. The crop although is known to be a self-pollinating one, the rates of out-crossing (7% to 91%) recorded by several investigators argue that *Capsicum* should be considered to be facultative crosspollinating species in field research [2, 3, 4, 5]. The pungency factor of pepper is one typical attribute for which all chillies are highly valued commercially. It is produced by capsaicinoids, the alkaloid compounds that are found only in the genus Capsicum [6]. Pepper sprays containing capsicum oleoresin provide ingredients for a non-lethal deterrent to some human and animal behavior and are useful riot control agents and self-defense tools [7].

Naga chilli or Bhut Jolokia (*C. chinense* Jacq.) which is endemic to north eastern part of India has been the centre of attraction owing to its characteristic unique aroma and a very high pungent flavour. This pepper had been declared as the hottest chilli in the world by Guinness World Records in 2006. Since then, it is constantly in news pertaining to its numerous pharmaceutical and therapeutic applications. Despite having tremendous commercial value, not much scientific progress has been made on it in terms of germplasm conservation so far. Moreover, the high capsaicin content has been deteriorating gradually due to uncontrolled cross-pollination and constant genetic mutation. In this context, it has become significantly important to retain the germplasm and maintain a constant level of capsaicin through biotechnological intervention, more precisely, micropropagation technique. Quite a good number of literatures is available so far on *in vitro* propagation of various *Capsicum* species. Several of these reports suggest a strong influence of genotype and media on the regeneration process [8, 9, 1, 10].

The function of silver nitrate (AgNO3) in the regeneration of micropropagated plantlets has been thoroughly elucidated in several crops including *Capsicum*. Addition of AgNO3 to the culture medium induced shoot regeneration in distal cotyledons of cucumber [11], enhanced callus development in *Brassica* [12] and improved embryogenic callus frequency in immature wheat embryos [13], symptoms like hyperhydricity and leaf epinasty caused by ethylene disappeared when potato cultivars were cultured in medium supplemented with AgNO3 [14].

It is by far known that AgNO3 is a potent anti ethylene compound. In *C. frutescens*, the combined effect of AgNO3 and CoCl2 caused extensive flower induction and shoot proliferation in in vitro raised plantlets [15]. AgNO3 has also been found to have profound effect on wheat anther culture [16].

Therefore, an experiment was designed, keeping the above points in view to standardize a regeneration protocol in Naga chilli and the functions of AgNO3 in combination with growth hormones have been examined throughout the process of *in vitro* organogenesis. The communication highlights on the effect of AgNO3 on *in vitro* flower and fruit induction in Naga chilli from leaf explants.

Materials and Methods

a. Explant Collection and Disinfection:

Young healthy leaves of Naga chilli were thoroughly treated with Bevistin solution (0.1% w/v) for 15-20 minutes. Followed by washing thoroughly with distilled water 4-5 times and later subjected to surface sterilization with 70% alcohol (5-10 sec). An aqueous solution of 0.1 (w/v) HgCl2 was prepared and leaves were further surface sterilized for 3 min. This was followed by rinsing leaf explants 4 -6 times with double distilled water to remove any traces of HgCl2. Leaf segments were cut aseptically in square pattern and inoculated onto Murashige and Skook's (MS) (Murashige and Skook, 1962) medium supplemented with growth hormones namely BAP (20-65µM/L), 2,4-D (1-5.5 µM /L) and AgNO3 (10-55 µM /L). The wounded ends were firmly dug onto the medium such that callus could develop from all four sides. The basal MS medium was augmented with 3% (m/v) sucrose (Himedia, Mumbai, India) and 0.8% (m/v) agar for solidification. The pH of all the media was maintained at 5.8 and after being autoclaved at 121°C and 15 pounds per square inch pressure for 15 minutes prior to inoculation. The cultures were aseptically incubated by maintaining a temperature of 25±2°C and kept under dark condition to facilitate callus formation.

b. Shoot Proliferation Medium

Green friable calli obtained from callus induction medium were carefully transferred onto fresh MS medium supplemented with plant hormones: BAP (20-65µM/L), 2,4-D (1-5.5 µM /L) and AgNO3 (10-55 µM /L) (Table 1). Healthy shoots that emerged from calli were further sub-cultured onto the medium with same hormonal composition at an interval of 3 weeks. These cultures were maintained at a temperature of 25±2°C and a photoperiod of 16 hour to encourage multiple shoot induction.

c. *In Vitro* Flower Induction and Fruit Formation

Healthy shoot buds selected from six-week-old cultures were transferred to MS medium enriched with/without GA3 (26-30 µM /L) and AgNO3 (30-34 µM /L) for *in vitro* flower induction. Flowering was observed approximately 35 days after inoculation. All terminal shoots bearing flowers were transferred delicately to fruit induction medium comprising of MS medium with different growth hormones as presented in Table 2. All cultures were subjected to 16/8 h light/dark cycle for fruit formation.

d. *In Vitro* Root Induction and Acclimatization

In vitro developed shoots were carefully transferred to rooting medium fortified with IBA (1.5-6.5 µM /L) and BAP (2-4.5 µM /L) (Table 3). These cultures were maintained at a temperature of 25±2°C and a photoperiod of 16 hour to stimulate root induction. After successful root initiation, plantlets were carefully taken out of the medium and washed delicately with tap water to remove any traces of hormones, agar and medium. For acclimatization of *in vitro* raised plantlets, sterile soil and vermicompost and vermiculite (1:1:1) were chosen and plantlets were carefully transferred to perforated plastic cups (to maintain appropriate humidity and kept in the polyhouse. Initially the cups were covered with polythene bags for 10-15 days. To maintain appropriate humidity, plantlets were watered once in three days with double distilled water. Later on, humidity was reduced by making small perforations in the polythene bags. Twelve-week-old hardened plants were eventually transferred to the open field. The percentage of plant survival was recorded.

Statistical Analysis

All the experiments were conducted with Complete Randomized Block Design (CRD) and experiments were repeated thrice with each treatment having 10 replicates. Significance between treatments was calculated using One-Way ANOVA and differences among different treatment means were based on Turkey's Honesty Significant difference (HSD at 0.05).

Result and Discussion (Paper 4)

a. Effect Of BAP, 2,4-D and AgNO3 on Callus Induction

As per Table 1, highest callus induction (47.1 ± 1.28) from the leaf explants Naga chilli was observed in treatment no. 6 with BAP, 2,4-D and AgNO3 at 45 µM/L 3.5 µM/L 35.0 µM/L respectively (Fig.1a). One of the well-known auxins for callus formation is 2,4-D. There are several reports highlighting the significance of 2,4-D in callus formation and shoot proliferation in various crops including *Capsicum* spp. According to Tahir *et al*., (2011) [17], yellowish compact nodular calli were generated from apical meristems of sugarcane cultivars, SP726180 and CO-001 when cultured in a modified MS medium supplemented with varying concentrations of 2,4-D. The authors reported that the increase in the rate of callus induction with the increase in doze of 2,4-D upto 4mg/L. Rashid *et al*. (2009) [18] who worked on wheat cultivars reported similar result where highest callus induction was obtained at a concentration of 3mg/L of 2,4-D. However, Malik *et al.* (2003) [19] observed that higher level of 2,4-D inhibited callus induction from mature wheat seeds. whereas lower concentration allows morphogenesis to occur. Role of AgNO3 on *in vitro* callus induction was also examined in the said investigation. As per the result rate of callus formation had been sharply increased in the presence of AgNO3. with an increase in the volume of undifferentiated tissues resulting in swelling and appearance of green friable callus. Similar type of observation was reported by Shah *et al*. (2014) [20] in Tomato.

b. Effect of Media Composition on *In Vitro* Multiple Shoot Induction

As per the data presented in Table 1, highest number of multiple shoots was obtained in treatment no 6 with BAP, 2,4-D and AgNO3 at 45 µM/L 3.5 µM/L 35.0 µM/L respectively (Fig.1b,c). Any alterations in the concentrations drastically reduced the number of multiple shoot induction. The shoots observed were lean and unhealthy when cultured on medium containing 2,4-D at concentration higher than 3.5 µM/L. BAP had been used as the sole cytokinin in the entire experiment. Significance of AgNO3 was highly observed. AgNO3 imparted positive response towards *in vitro* shoot proliferation and percentage of shoot induction. Concentration of AgNO3 for *in vitro* shoot induction was standardized at 3.5 µM/L. AgNO3 has been reported as a potent ethylene inhibitor by Sharma *et.al*. (2008) [15] Their report stated that silver ions protect the plants from senescence caused by ethylene thus preventing the shoots from falling off. Hence, it is an essential component for the process of induction and elongation of shoots in pepper.

Absence of AgNO3 remarkably reduced the number of multiple shoots in the said experiment. Similar type of findings were reported in tomato by Sheeja and Mandal (2003) [21]; Anantasaran and Kanchanapoom (2008) [22] in Zinnia, Ashrafuzzaman *at al*. in *Capsicum* species (2009) [23] and Sandra and Maira (2013) [24] in potato.

c. Effect of Media Composition on *In Vitro* Flower and Fruit Induction

As evident from the data shown in Table 2, combination of GA3 and AgNO3 played an important role in producing maximum number of flower and fruit under *in vitro* conditions. Maximum induction of flower per shoot (6.6) was recorded in the treatment where AgNO3 at 34µM/L was combined with GA3 at 28µM/L (Fig.1e). Petals were of off-white colour which remained healthy for two weeks. Withdrawal of AgNO3 from the medium would reduce drastically the number of flower buds. This clearly signifies the importance of AgNO3 on *in vitro* flower induction. AgNO3 has been reported Sharma *et al*., (2008) [15] to inhibit ethylene action. The exact mechanism of AgNO3 mediated ethylene production and its activity regulation is unclear but it has been explained by an interference of ethylene perception or stress exerted by silver ion. [21] Silver nitrate is an ethylene action inhibitor and ethylene inhibits S-adenosyl methionine decarboxylase, which in turn promotes polyamine levels, which are implicated in flowering (Bais *et al*. (2000) [25] and Sharma *et al*. (2008) [15]. Coming to *in vitro* fruit formation, a total of 7.5 fruits generated from a single explant in the same combination (Fig.1f). GA3 is known to play important role in fruit development in many plant species. The said investigation complies with the report submitted by Buzzy *et al*., (2005) [26] where the authors suggested possible positive influence of GA3 on in vitro regeneration of Hebenero pepper via organogenesis.

d. Effect of Media Composition on *In Vitro* Root Induction and Acclimatization

For *in vitro* root initiation, IBA was chosen over IAA and NAA in combination with BAP. Initially the experiment was conducted with IAA and NAA together and in combinations. However, roots developed under such combinations were found to be very lean and thin which did not survive more than a week. As per figures shown in Table 3, maximum *in vitro* root induction per shoot was observed in the medium supplemented with IBA (4.5µM/L) and BAP (3.5µM/L) (Fig.1d). A highest of 61 roots/explant was formed within 2 weeks after being transferred to rooting medium.

The *in vitro* raised plantlets of Naga chilli were carefully transferred to poly house to serve the purpose. Substrates containing sterile soil, vermicompost and vermiculite (1:1:1) generated best results. Percentage of plant survival was 86.65%. According to report submitted by Azad *et al*., (2011) [27], organic farming supplemented with of biofertilizers in the long-run can be considered an important contributor to food security. As per another findings by Bhat *et al*., (2013) [28] use of good quality compost and biofertilizers stimulate the activity of heterotrophic microbes present in the rhizosphere region where it mineralize nutrients, particularly nitrogen in the incorporated organic fertilizers, thus making them available to the plants. In addition to this, the report also says that it improves soil texture, reduces bulk density and increases the available water content.

Conclusion

In the said experiment, the importance of AgNO3 on *in vitro* regeneration of Naga chilli has been elucidated. As per the findings, AgNO3 played an important role mostly in reproductive stage of flower and fruit induction. Naga chilli, being one of the hottest peppers in the world, has tremendous economic and research potential as food components, medicines, and pharmaceuticals. However, not much micropropagation has been conducted so far. Therefore, it is believed that the said investigation would pave a way out to get into the insights of micropropagation techniques in this pungent chilli pepper of north east India.

Fig 1: (a) *In vitro* callus induction (b) and (c) Multiple shoot induction d) *In vitro* root induction (e) *In vitro* flower induction (f) *In vitro* fruit induction (g) and (h) Acclimatization of *in vitro* raised plantlets

Table 1: Effect of various media composition on *in vitro* regeneration, multiple shoot induction and shoot length of leaf explants of Naga Chilli (*Capsicum chinense*)

Sl.No.	Treatments MS+BAP+ 2,4-D + AgNO3 (µM/L)	No.of callus Mean±SE	No. of multiple shoots/callus Mean±SE	Shoot length (cm) Mean±SE
1	20.0+1.0+10.0	11.0±2.12a	3.6 ± 0.69a	2.6± 0.53a
2	25.0+1.5+15.0	19.4 ± 2.47b	3.8 ± 0.41a	4.4± 0.22b
3	30.0+2.0+20.0	25.3± 2.93c	5.6 ± 0.63b	5.4 ± 0.54c
4	35.0+2.5+25.0	31.7± 4.13d	5.7 ± 0.43b	6.7 ± 0.39d
5	40.0+3.0+30.0	44.4 ± 1.49e	6.8± 0.67c	6.9 ± 0.37d
6	45.0+3.5+35.0	47.1 ± 1.28f	8.0 ± 0.74d	6.0± 0.42e
7	50.0+4.0+40.0	41.9 ± 2.76g	6.9 ± 1.40c	5.6± 0.37c
8	55.0+4.5+45.0	36.6 ± 3.23h	6.8 ± 1.60c	5.0± 0.63f
9	60.0+5.0+50.0	32.8± 4.02d	5.2 ± 1.33b	4.3 ± 0.49b
10	65.0+5.5+55.0	26.5 ± 4.37c	5.0 ± 0.46b	3.9 ± 0.60g

Means followed by the same letters are not significantly different at p≤0.05

Table 2: Effect of different concentrations of GA3 and AgNO3 on *in vitro* flower and fruit induction of leaf explants of Naga Chilli (*C. chinense)*

Sl.No.	Treatments MS+GA3+ AgNO3 (µM/L)	No. of flower buds/explant Mean±SE	No. of flowers/explant Mean±SE	No. of fruits/explant Mean±SE
1	26 + 0	7.4±1.25a	4.1 ± 0.95a	3.4 ± 0.95a
2	30 + 0	10.3±1.13b	7.2 ± 0.97b	6.2± 0.73b
3	28 + 34	13.5±1.35c	6.6 ± 1.04c	7.5 ± 1.51c
4	0 + 32	10.5±1.73b	7.0± 1.27b	5.3± 1.38d
5	0 + 30	7.8 ± 1.03a	4.5± 1.03a	3.1 ± 0.69a

Means followed by the same letters are not significantly different at p≤0.0

Table 3: Effect of different concentrations of BAP and 2,4-D on *in vitro* root induction and root length of leaf explants of Naga Chilli (*C. chinense*)

Sl.No.	Treatments MS+BAP+IBA (µM/L)	No. of roots/regenerated shoot Mean±SE	Root length (cm) Mean±SE
1	2.0+1.5	25.3±4.93a	4.5 ± 1.27a
2	2.5+2.5	31.1±6.50b	6.7 ± 0.86b
3	3.0+3.5	46.3±7.18c	8.8 ± 1.66c
4	3.5+4.5	61 .0±7.43d	15.6± 3.18d
5	4.0+5.5	55.3 ± 7.55e	17.63 ± 2.46e
6	4.5+6.5	48.3 ± 3.32c	14.5 4± 2.70f

Means followed by the same letters are not significantly different at p≤0.05

References

1. Sanatombi K, Sharma GJ. Micropropagation of Capsicum annum L. using axillary shoot explants. Sci Hort,2007:113:96-99
2. Bosland PW. Capsicums: Innovative uses of an ancient crop. In: Janick J (ed.), Progress in new crops. Arlington, 1996, 479-487.
3. Odland ML, Porter AM. A study of natural crossing in pepper (Capsicum frutescens L.). J. Am Soc Hort Sci,1941:38:585-588.
4. Franceschetti U. Natural cross pollination in pepper (Capsicum annuum L.). Proc. Eucarpia Meeting on Genetic and Breeding of Capsicum. Turin, Italy, 1971, 346-353.
5. Tanksley SD. High rates of cross-pollination in chile pepper. Hort Sci,1984:19:580-582.
6. Bosland PW, Votava EJ. Peppers: vegetables and spice Capsicums. Crop Production Science in Horticulture. England,2000:12:204.
7. De AK. Capsicum: The Genus Capsicum. Medicinal and aromatic plants. Industrial Profiles Taylor & Francis, London and New York,2003:33:275.
8. Ramage CM, Leung DWM. Influence of BA and sucrose on the competence and determination of Pepper (Capsicum annum L var. Sweet Banana) hypocotyl cultures during shoot formation. Plant Cell Rep,1996:15:974-979.
9. Franck-Duchenne M, Wang Y, Tahar SF, Beachy RN. In vitro stem elongation of sweet pepper in media containing 24-epi-brassinolide. Plant Cell Tiss Org Cult,1998:53:79-84.
10. Mihalka V, Fari M, Szasz A, Balazs E. Optimized protocols for efficient regeneration and gene transfer in pepper (Capsicum annuum L.). J Plant Biotechnol,2000:2:143- 149.
11. Mohiuddin AKM, Chowdhury MKU, Zaliha C, Abdullah, Napis S. Influence of silver nitrate (ethylene inhibitor) on cucumber in vitro shoot regeneration. Plant Cell Tiss Org Cult,1997:51:75-78.
12. Williams J, Pink DAC, Biddington NL. Effect of silver nitrate on longterm culture and regeneration of callus from Brassica oleracea var. gemmifera. Plant Cell Tiss Org Cult,1990:21:61-66.
13. Wua LM, Weia YM, Zhenga YL. Effects of silver nitrate on the tissue culture of immature wheat embryos. Rus J Plant Physiol,2006:53:530- 534.
14. Sandra AT, Maira O. Effect of culture medium consistence and silver nitrate on micropropagation of two potato (Solanum tuberosum) cultivars. Rev Colomb Biotechnol,2013:2:55-62.

15. Sharma A, Kumar V, Giridhar P, Ravishankar GA. Induction of in vitro flowering in Capsicum frutescens under the influence of silver nitrate and cobalt chloride and pollen transformation. Electr J Biotech,2008:11:1-6
16. Hasan MF, Islam SMS. Effect of silver nitrate and growth regulators to enhance anther culture response in wheat (Triticum aestivum L.) Heliyon, 2021, 2405-8440
17. Tahir SM, Victor K, Abdulkadir S. The Effect of 2, 4- Dichlorophenoxy Acetic Acid (2, 4-D) concentration on callus induction in sugarcane (Saccharum officinarum). Nigerian Journal of Basic and Applied Science,2011:19(2):213-217
18. Rashid U, Ali S, Muhammad G, Ayub AN, Masood MS. Establishment of an efficient callus induction and plant regeneration system in Pakistani wheat (Triticum aestivum) cultivars. Electronic Journal of Biotechnology, 2009, 12(3).
19. Malik SI, Rashid H, Yasmin T, Minhas NM. Effect of 2,4- dichlorophenoxyacetic Acid on Callus Induction from Mature Wheat (Triticum aestivum L.) Seeds. International Journal of Agriculture & Biology,2003:6(1):156-159.
20. Shah SH, Ali S, JAN SA, Jalal-ud–din, Ali GM. Assessment of silver nitrate on callus induction and in vitro shoot regeneration in tomato (Solanum lycopersicum mill.) Pakistan Journal of Botany,2014:46(6):2163-2172.
21. Sheeja TE, Mandal AB. In vitro flowering and fruiting in tomato (Lycopersicon esculentum Mill.). Asia Pacific Journal of Molecular Biology and Biotechnology,2003:11:37-42
22. Anantasaran J, Songklanakarin KK. Influence of medium formula and silver nitrate on in vitro plant regeneration of Zinnia cultivars. J. Sci. Technol,2008:30:1-6
23. Ashrafuzzaman M, Hossain MM, Ismail MR, Haque MS, Shahidullah SM, Zaman S. Regeneration potential of seedling explants of chilli (Capsicum annuum). African J of Biotechnology,2009:8:591-596.
24. Sandra AT, Maira O. Effect of culture medium consistence and silver nitrate on micropropagation of two potato (Solanum tuberosum) cultivars. Rev. Colomb. Biotecnology,2013:2:55-62.
25. Bais HP, Sudha GS, Ravishankar GA. Putrescine and AgNO3 influences shoot multiplication, in vitro flowering and endogenous titers of polyamines in Cichorium intybus L. cv. Lucknow Local. Journal of Plant Growth Regul,2000:19:238-248.
26. Buzzy NS, Flick AC, Perez FB, Peniche MCM, Castillo PYZ., Ruiz AS., Colli AZ., Alonso OG. and Ham MLM. Regeneration of Hebenero pepper (Capsicum chinense J.) via organogenesis. HortSci,2005:40:1829-1831.
27. Azad H, Schoonbeek S, Mahmoudi H, Derudde B, Maeyer PD, Witlox F. Organic agriculture and sustainable food production system: Main potentials. Agric. Ecosyst. Environ,2011:144:92-94.
28. Bhat TA, Gupta M, Ganai MA, Ahanger RA, Bhat HA. Yield, soil health and nutrient utilization of field pea (Pisum sativum L.) as affected by phosphorus and biofertilizers under subtropical conditions of Jammu. International Journal of Modern Plant and Animal Science,2013:1:1-8.