

## Original Research Article

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## Callus Mediated *In vitro* Regeneration of Naga Chilli (*Capsicum chinense* Jacq.): The Fiery Pepper from North East India

G. Bora<sup>1,2\*</sup>, H. K. Gogoi<sup>1</sup> and P. J. Handique<sup>2</sup><sup>1</sup>Division of Biotechnology, Defence Research Laboratory, Tezpur-784001, Assam, India<sup>2</sup>Department of Biotechnology, Gauhati University, Guwahati-781014, India

\*Corresponding author

### ABSTRACT

Naga chilli or Bhut jolokia is extensively cultivated all over northeast India including Assam. This chilli is of high commercially value due to its pungent trait called capsaicin. The chilli is well known to have extreme potential for numerous pharmaceutical applications apart from being used for culinary purposes. Despite being such an important spice crop, demand of Naga chilli is deteriorating owing to natural cross pollination and mutation which in turn reduce its capsaicin content. Therefore, an attempt was made to conserve the germplasm through standardization of an efficient micropropagation protocol using different plant hormones, silver nitrate (AgNO<sub>3</sub>) and tryptophan as the key components. *In vitro* propagation was carried out with leaf explants. MS medium was supplemented with different combinations of BAP, 2,4-D, GA<sub>3</sub>, NAA and IBA alongside AgNO<sub>3</sub> and tryptophan. Maximum callus induction took place in the presence of 2,4-D (3.5mg/L), AgNO<sub>3</sub> (3mg/L) and trptophan (3mg/L). Addition of AgNO<sub>3</sub> to the culture medium greatly enhanced shoot proliferation in the selected *in vitro* plantlets. *In vitro* flower induction and fruit formation were successfully carried out in the presence of GA<sub>3</sub> (3.5mg/L) and AgNO<sub>3</sub> (3.5mg/L). Maximum root initiation was observed in the MS medium supplemented with IBA (3mg/L), GA<sub>3</sub> (2.5mg/L) and AgNO<sub>3</sub> (3mg/L). Hardening of *in vitro* raised plantlets was successful in sterile soil enriched with vermicompost and vermiculite. Percentage of plant survival was 84.75%. Addition of biofertilizers greatly improved the chances of acclimatization of *in vitro* raised healthy plantlets.

#### Keywords

Naga chilli, *In vitro* propagation, Silver nitrate, tryptophan

#### Article Info

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### Introduction

Chilli pepper belongs to the genus *Capsicum* (Family: Solanaceae) which represents one of the diverse plant groups. The genus *Capsicum* consists of approximately 25 wild and 5 domesticated species (Sanatombi and Sharma, 2007). Out of the five domesticated species (*C. annum* L., *C. chinense* Jacq., *C. frutescens* L., *C. baccatum* L., and *C.*

*pubescens* R&P), first three species namely, *C. annum* L., *C. chinense* Jacq., *C. frutescens* L. are broadly cultivated in our country. Naga chilli or Bhut Jolokia which is endemic to northeastern part has gained much attention by the world scientific community owing to its characteristic unique aroma and high pungent flavour. In 2006, this pepper had been declared as the hottest chilli in the world by Guinness World Records. Since then it is

constantly in news pertaining to pharmaceutical and therapeutic applications. It is known by various names in different regions such as 'Bhut jolokia' or 'Bih jolokia' in Assam, 'Naga king chilli' in Nagaland, 'Omorok' in Manipur and 'Ghost pepper' by the western media. It is also known by the names, 'Saga jolokia', 'Indian mystery chilli' and 'Indian rough chilli' (after the chilli's rough skin) (Meghvansi *et al.*, 2010). The one attribute most typical of chillies is its pungency. The pungency factor present in the *Capsicum* fruit are predominantly capsaicin and dihydrocapsaicin contributing up to 80–90% of total capsaicinoids. The pharmaceutical applications of capsaicinoids are attributed to their analgesic, anti-arthritic, anticancer, and antioxidant properties (Prasad *et al.*, 2005). Prasad *et al.*, (2005) reviewed that capsaicin has been at the centre of intense research for elucidating the basis of its pharmacological properties and exploiting the therapeutic potential. Moreover capsaicin has become a promising molecule for the development of a new generation of analgesic-anti-inflammatory agents targeting the nociceptive primary afferent neurons (Szolcsanyi, 2003). The antioxidative capacity of chilli is higher than ginger, garlic, mint and onion (Shobana and Naidu, 2000), which may play an important role in the process of chemo- prevention according to Yu *et al.*, (2002). Capsaicin has also been reported to inhibit the growth of prostate cancer cells (Mori *et al.*, 2006). In addition to that capsaicin is being used to alleviate pain. Its mode of action is thought to be from nerve endings releasing a neurotransmitter called substance P (Bosland, 1996). Despite being therapeutically important due to high capsaicin content, demand of Naga chilli is under question as very little scientific progress has been made. Moreover, the extent of hotness seems to be decreasing due to rapid cross pollination and mutation. Therefore, in this context, it is considered as important as to

conserve the original germplasm thus maintaining a constant level of capsaicin through biotechnological intervention, more precisely, micropropagation or tissue culture technique.

A number of literatures are available on *in vitro* propagation of various *Capsicum* species. In *capsicum*, direct regeneration is the most preferred type of regeneration reported from various explants however few authors claim indirect plant regeneration (through callus culture) to be a better option. Role of silver nitrate ( $\text{AgNO}_3$ ) has been cited in *in vitro* regeneration of several crops including cucumber (Mohiuddin *et al.*, 1997), *Brassica* (Williams *et al.*, 1990), wheat (Wua *et al.*, 2006) and *C. frutescens* (Sharma *et al.*, 2008). Function of tryptophan has also been elucidated in various reports: neem (Gehlot *et al.*, 2014), rice (Shahsavari, 2011) and *Catharanthus roseus* (Rahmatzadeh *et al.*, 2014). Keeping the above points in mind, an attempt has been made to obtain *in vitro* organogenesis in Naga chilli through leaf derived callus culture under the influence of various plant growth hormones and two key components,  $\text{AgNO}_3$  and tryptophan.

## Materials and Methods

### Explant Collection and Disinfection

For indirect regeneration via callus phase, healthy young leaves excised from *C. chinense* variety 'Naga chilli' maintained in the Division of Biotechnology at Defence Research Laboratory (DRL), Tezpur, Assam (India) were treated with 0.1% Bevistin solution (w/v) for 15-20 minutes. Leaves were washed thoroughly with distilled water 4-5 times and later subjected to surface sterilization by with 70% alcohol (5-10 sec), followed by a treatment with an aqueous solution of 0.1 (w/v)  $\text{HgCl}_2$  for 3 min. This was followed by rinsing leaf explants 4 -6

times with double distilled water to remove any traces of HgCl<sub>2</sub>. Leaf segment was cut aseptically from all sides in square pattern and inoculated onto Murashige and Skoog's (MS) (Murashige and Skoog, 1962) medium supplemented with growth hormones namely BAP (1.5mg/L), 2,4-D (1.5-5.0mg/L), AgNO<sub>3</sub> (1.0-4.5mg/L) and Tryptophan (1.0-4.5mg/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 autoclaved at 121°C and 15 pounds per square inch pressure for 15 minutes prior to inoculation. The cultures were incubated at a temperature of 25±2°C and kept under dark condition to facilitate callus formation.

### **Shoot proliferation**

Green friable calli were transferred onto fresh MS medium supplemented with plant hormones: BAP (1.5-5.0mg/L), NAA (1.5mg/L) AgNO<sub>3</sub> (2.0-5.5mg/L) and tryptophan (1.5mg/L). Healthy shoots that emerged from calli were sub-cultured thrice onto the medium with same hormonal composition at an interval of 3 weeks. These cultures were maintained at 25±2°C and a photoperiod of 16 hour to encourage multiple shooting.

### **In Vitro Flower Induction and Fruit Development**

Flower induction under *in vitro* condition was achieved by shifting the healthy shoot buds selected from six-week-old cultures onto MS medium supplemented with BAP (1.5mg/L), enriched with/without GA<sub>3</sub> (2.0-3.5-mg/L) and AgNO<sub>3</sub> (2.0-3.5mg/L). *In vitro* flowering was observed after 35 days of inoculation. All terminal shoots bearing flowers were transferred delicately to fruit induction

medium comprising of MS medium with different growth hormones as presented in Table3. All cultures were subjected to 16/8 h light/dark cycle for fruit formation.

### **In Vitro Root Induction and Acclimatization**

*In vitro* developed shoots were carefully transferred to rooting medium supplemented with IBA (1.5-5.0mg/L), BAP (1.5mg/L), GA<sub>3</sub> (2.5mg/L), and AgNO<sub>3</sub> (1.5-5.0mg/L). Culture conditions were kept as same as described earlier for *in vitro* organogenesis. After successful root initiation, plantlets were carefully taken out of the medium and washed delicately under tap water to remove any traces of hormones, agar and medium. For hardening and acclimatization of *in vitro* raised plantlets, plastic cups containing sterile soil and vermicompost and vermiculite (1:1:1) were chosen and plantlets were carefully transferred to these cups and kept in the polyhouse.

The cups were covered with polythene bags for the first 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 acclimatized plants were eventually transferred to the open field. The percentage 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 Honest Significant difference (HSD at 0.05).

## Results and Discussion

### Effect of 2,4-D, AgNO<sub>3</sub> and tryptophan on callus induction

From the data shown in Table 1, highest callus induction from the leaf explants of *C. chinense* variety, Naga chilli (in terms of number) was observed in the treatment containing 2,4-D (3.5mg/L), AgNO<sub>3</sub> (3.0mg/L), tryptophan (3mg/L) along with BAP at a fixed concentration of 1.5mg/L. To obtain friable green callus, the concentration of BAP was maintained at 1.5mg/L. BAP at higher dose (>1.5mg/L), led to poor callusogenesis. As far the percentage of callus induction was concerned, callusogenesis was best obtained at a concentration slightly higher than the former treatment. A highest of 86.34% of callus induction took place in MS medium supplemented with BAP (1.5mg/L), 2,4-D (4mg/L) and fortified with AgNO<sub>3</sub> and tryptophan at a concentration of 3.5mg/L each. One of the well-known auxins for callus formation is 2,4-D. There are several reports citing the importance of 2,4-D in callus formation and shoot proliferation in various crops including *Capsicum* spp. According to Tahir *et al.*, (2011), 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 further stated that the rate of callus induction increased with the increase in dose of 2,4-D upto 4mg/L. The production of a yellowish, compact and nodular callus at cut edge of explant may be due to the wound caused during the process of cutting which resulted in a synchronous cell division. This is considered as a process of de-differentiation of organized tissue (Tahir *et al.*, 2011). Similar observation was also reported in by Rashid *et al.*, in 2009 in wheat cultivars. As per their report, highest callus induction was obtained at a

concentration of 3mg/L of 2,4-D. On the contrary, Malik *et al.*, in 2003 observed that callus induction of mature wheat seeds was significantly inhibited at higher level of 2,4-D whereas lower concentration allows morphogenesis to occur. Another significant observation was made in sweet potato by Bett *et al.*, (2015) confirming that highest percentage of callusogenesis from leaf explants was obtained at 2mg/L of 2,4-D whereas stem explants generated best results at quite a higher range of 5mg/L. Response of *in vitro* raised plantlets towards plant hormones depends upon type of explants and genotype of the plant in concern. Various studies reveal that induction of callus from leaf, hypocotyls or cotyledonary explants of *Capsicum* spp. respond best under the influence of 2,4-D than any other forms of auxin (Umameshwari and Lalitha, 2007; Rao and Sangapure, 2014 and Suthar and Shah, 2015). In the present investigation, the average duration for callus formation from leaf explants was after 8<sup>th</sup> day of inoculation. However, the duration seemed to increase upon increasing the concentration beyond 4mg/L. Similar observation was also reported by Hasnat *et al.*, (2007) in *C. frutescens*. The authors in their report stated that availability of auxin in optimum concentration is required for activation of expansin enzyme for explant cell wall loosening and extensibility leading to increase in initial growth of explants. On the other hand, delayed callus initiation at low concentrations of 2,4-D might have occurred owing to reduced enzyme activity of RNA polymerase involved in growth processes whereas higher concentrations may lead to extra and abnormal growth of explant by preventing conversion of immature cytoplasm to mature cytoplasm (Taiz & Zeiger, 2002).

In addition to that, Davletova *et al.*, (2001) reported that 2,4-D exerted the primary control on endogenous synthesis and metabolism of IAA and cytokinin in cells

which played significant role in the process of callus induction. Role of  $\text{AgNO}_3$  and tryptophan in *in vitro* callus induction was also assessed in the said investigation. Our experimental findings revealed that rate of callus formation abruptly increases in the presence of these two key components. Addition of  $\text{AgNO}_3$  caused an increase the volume of undifferentiated tissues resulting in swelling and appearance of green friable callus. Similar observation was confirmed in tomato by Shah *et al.*, in 2014. Similarly in another experiment, Srichuay and Te-chato (2012) obtained maximum callus induction when explants of *Hevea brasiliensis* were inoculated in MS medium supplemented with 1mg/L of  $\text{AgNO}_3$ . Another important source of nutrition is tryptophan. Tryptophan being an auxin precursor helps in the process of *in vitro* morphogenesis. In the present investigation, maximum callusogenesis took place in the treatment that contained 3.5mg/L of tryptophan. At doses higher or lower than the said value, callus induction was seen to be affected severely. In upland rice, similar findings were reported by Shahsavari (2011). However, there is no any report so far in regard to tryptophan effect in Naga chilli.

### **Effect of media composition on *in vitro* multiple shoot induction**

For any *in vitro* process to become successful the most important step to be taken care of is shoot proliferation. Our investigation was aimed to obtain highest number of multiple shoot out of different hormonal combinations from each callus regenerated in the previous experiment. From the data shown in Table 2, highest number of multiple shoots (8.95) was obtained with treatment  $T_5$  where BAP (3.5mg/L) and NAA (1.5mg/L) were combined with  $\text{AgNO}_3$  and tryptophan at 4mg/L and 1.5mg/L respectively. Slight alteration in their concentrations would drastically affect multiple shoot induction. The

shoots observed were lean and unhealthy when cultured on medium containing NAA at concentration higher than 1.5mg/L. This result doesn't comply with the findings of Song *et al.*, (2010). Authors stated that NAA at higher concentration exerts positive impact not only on shoot proliferation but also percentage of shoot regeneration. However, in the present study, highest shoot induction percentage (81.32%) was achieved at 1.5mg/L NAA. This discrepancy might have occurred owing to differences in responsiveness of pepper genotypes. Moreover, excessive auxin concentration might have caused endogenous hormonal imbalance that would reduced drastically shoot length and proliferation. As far shoot length is concerned, longest shoot of 5.97cm was obtained in  $T_4$  (3mg/LBAP, 1.5mg/LNAA, 3.5mg/L  $\text{AgNO}_3$  and 1.5mg/L tryptophan)

Entire investigation was conducted with BAP as the sole cytokinin. Plantlets were initially treated with thidiazuron (TDZ). But later it was withdrawn from the culture medium as the plantlets cultured on medium supplemented with TDZ (with/without auxin) were found short with frail shoots which would easily fall off. Our observation complies with the findings of Ostrosky *et al.*, (2011). But the other reports confirmed positive response of TDZ towards shoot induction and proliferation of different *Capsicum* species (Song *et al.*, 2010; Buzzy *et al.*, 2005 and Kehia *et al.*, 2012).

Role of  $\text{AgNO}_3$  was examined. Several sets of experiments were conducted with  $\text{AgNO}_3$ . Table 2 signifies positive response of  $\text{AgNO}_3$  towards *in vitro* shoot proliferation and percentage of shoot induction. Concentration of  $\text{AgNO}_3$  for *in vitro* morphogenesis was optimized at 4mg/L. However, at slightly higher dose (4.5mg/L), percent shoot regeneration was maximum (81.32%).  $\text{AgNO}_3$  is a potent ethylene inhibitor.



**Table.1** Effect of phytohormones, AgNO<sub>3</sub> and tryptophan on number of *in vitro* callus induction and percentage of callus regeneration in Naga chilli

Treatment	Treatment composition MS+hormones(mg/L)				Callus induction (No.)	Regenerated callus (%)
	BAP	2,4-D	AgNO <sub>3</sub>	Trp		
T <sub>1</sub>	1.5	1.5	1.0	1.0	17.41	47.12
T <sub>2</sub>	1.5	2.0	1.5	1.5	22.23	56.43
T <sub>3</sub>	1.5	2.5	2.0	2.0	28.35	66.46
T <sub>4</sub>	1.5	3.0	2.5	2.5	34.54	78.21
T <sub>5</sub>	1.5	3.5	3.0	3.0	42.12	82.76
T <sub>6</sub>	1.5	4.0	3.5	3.5	38.56	86.34
T <sub>7</sub>	1.5	4.5	4.0	4.0	31.78	71.65
T <sub>8</sub>	1.5	5.0	4.5	4.5	26.32	64.98
S.Ed(±)					1.92	1.13
C.D. at 5%					2.13	1.67

**Table.2** Effect of phytohormones, AgNO<sub>3</sub> and tryptophan on *in vitro* shoot proliferation, shoot length and percentage of shoot regeneration in Naga chilli

Treatment	Treatment composition MS+hormones(mg/L)				Multiple shoot induction (No)	Shoot Length (cm)	Regenerated shoot (%)
	BAP	NAA	AgNO <sub>3</sub>	Trp			
T <sub>1</sub>	1.5	1.5	2.0	1.5	2.65	2.15	33.76
T <sub>2</sub>	2.0	1.5	2.5	1.5	3.97	2.97	47.52
T <sub>3</sub>	2.5	1.5	3.0	1.5	4.65	3.65	52.87
T <sub>4</sub>	3.0	1.5	3.5	1.5	6.34	5.97	68.62
T <sub>5</sub>	3.5	1.5	4.0	1.5	8.95	4.93	75.93
T <sub>6</sub>	4.0	1.5	4.5	1.5	8.19	4.00	81.32
T <sub>7</sub>	4.5	1.5	5.0	1.5	7.00	3.06	73.16
T <sub>8</sub>	5.0	1.5	5.5	1.5	5.52	2.00	63.00
S.Ed(±)					1.64	0.52	1.97
C.D. at 5%					1.93	0.64	2.74

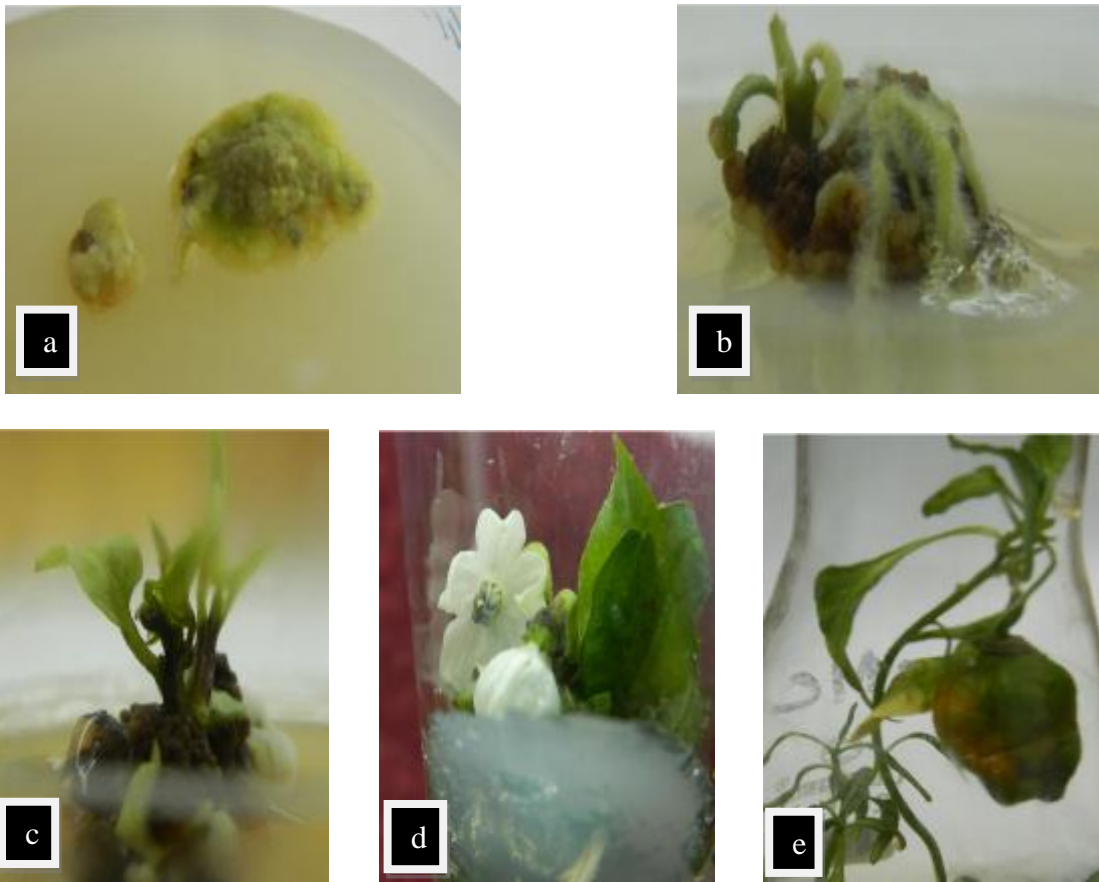
**Table.3** Effect of phytohormones, AgNO<sub>3</sub> and GA<sub>3</sub> on *in vitro* flower induction, and fruit formation in Naga chilli

Treatment	Treatment composition MS+hormones(mg/L)			No. of <i>in vitro</i> flower induction	No. of <i>in vitro</i> fruit formation
	BAP	GA <sub>3</sub>	AgNO <sub>3</sub>		
T <sub>1</sub>	1.5	2.0	0.0	2.73	2.71
T <sub>2</sub>	1.5	2.5	0.0	4.61	3.96
T <sub>3</sub>	1.5	3.0	0.0	5.21	4.21
T <sub>4</sub>	1.5	3.5	0.0	4.10	6.75
T <sub>5</sub>	1.5	0.0	2.0	3.42	2.54
T <sub>6</sub>	1.5	0.0	2.5	5.96	3.64
T <sub>7</sub>	1.5	0.0	3.0	6.84	4.83
T <sub>8</sub>	1.5	0.0	3.5	8.42	3.85
S.Ed(±)				1.22	1.05
C.D. at 5%				2.87	1.92

**Table.4** Effect of phytohormones and AgNO<sub>3</sub> on *in vitro* root induction, root length and percentage of root regeneration in Naga chilli

Treatment	Treatment composition MS+hormones(mg/L)				Multiple root induction (No)	Root Length (cm)	Regenerated root (%)
	BAP	IBA	GA <sub>3</sub>	AgNO <sub>3</sub>			
T <sub>1</sub>	1.5	1.5	2.5	1.5	19.54	9.43	62.56
T <sub>2</sub>	1.5	2.0	2.5	2.0	24.59	14.32	71.43
T <sub>3</sub>	1.5	2.5	2.5	2.5	32.54	16.53	74.56
T <sub>4</sub>	1.5	3.0	2.5	3.0	46.65	14.59	80.96
T <sub>5</sub>	1.5	3.5	2.5	3.5	42.54	15.34	79.43
T <sub>6</sub>	1.5	4.0	2.5	4.0	37.43	12.42	74.23
T <sub>7</sub>	1.5	4.5	2.5	4.5	33.43	12.96	70.39
T <sub>8</sub>	1.5	5.0	2.5	5.0	29.74	10.53	69.42
S.Ed(±)					1.04	0.38	1.64
C.D. at 5%					1.95	1.01	2.12

**Fig.1** *In vitro* propagation of *C. chinense* variety Naga chilli (a) Callus induction (b) Shoot regeneration (c) Multiple shoot induction (d) Flower Induction (e) Fruit Formation (f) Root induction (e) *Ex vitro* acclimatization (Soil+vermicompost+vermiculite in 1:1:1 ratio)





It is found to be an essential component for the process of induction and elongation of shoots in pepper. Absence of  $\text{AgNO}_3$  remarkably reduced the number of multiple shooting. Silver ions protect the plants from senescence caused by ethylene thus preventing the shoots from falling off. Similar trend was noticed in tomato by Sheeja and Mandal (2003); Anantasaran and Kanchanapoom (2008) in *Zinnia*, Ashrafuzzaman *et al.* in *Capsicum* species (2009) and Sandra and Maira (2013) in potato.

We were not able to see any significant impact of tryptophan in the process of shoot proliferation in our investigation. Tryptophan unlike  $\text{AgNO}_3$  failed to show any positive response in shoot elongation and multiplication. Tryptophan is an Indole-3-acetic acid IAA (auxin) precursor.

An auxin is preferred more in case of callus induction and rooting than shoot induction. Keeping the above points in mind the concentration of tryptophan was therefore, fixed at 1.5mg/L throughout the experiment.

Shahsavari in 2011 stated that addition of tryptophan to the medium not only increases the rate of *in vitro* callus induction but also enhances the chances of shoot proliferation in upland rice. However, in our investigation, this trend was not followed.



### **Effect of media composition on *in vitro* flower and fruit induction**

Reproductive phase is the most decisive phase in the life cycle of a plant. The phase becomes more crucial when it comes to *in vitro* regeneration. An attempt was made in Naga chilli to focus on morphogenic development leading to flower and fruit induction under the influence of  $\text{AgNO}_3$  and  $\text{GA}_3$ . Data shown in Table 3 represent direct correlation of plant growth hormones with *in vitro* organogenesis, *i.e.* flower induction and fruit formation. The investigation was carried out with BAP,  $\text{AgNO}_3$  and  $\text{GA}_3$ . BAP concentration was maintained at 1.5mg/L throughout the experiment. Two sets of experiments were conducted. First four treatments ( $T_1$ ,  $T_2$ ,  $T_3$ ,  $T_4$ ) comprised of BAP and  $\text{GA}_3$  and the last four ( $T_5$ ,  $T_6$ ,  $T_7$ ,  $T_8$ ) BAP and  $\text{AgNO}_3$ . As per the data shown in Table 3 maximum production of flower per shoot (8.42) was recorded in the treatment where  $\text{AgNO}_3$  at 3.5mg/L was combined with 1.5mg/L of BAP. All flowers were of off-white colour which remained open for 14 days. Withdrawal of  $\text{AgNO}_3$  from the medium would reduce drastically the number of flower buds and the value dropped down to 2.73, which was quite a disappointing figure. This clearly reflects the importance of  $\text{AgNO}_3$  on its reproductive flowering stage.  $\text{AgNO}_3$  has been reported to inhibit ethylene action (Beyer, 1976 and Sharma *et al.*, 2008). The exact mechanism of



AgNO<sub>3</sub> 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 (Sharma *et al.*, 2008). 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 and Sharma *et al.*, 2008). It has been proved beyond doubt that polyamines play crucial roles in plant growth and development as well as basic biological process (reviewed by Kumar and Rajam, 2004 and Kumar *et al.*, 2009). Since polyamines have been reported to promote embryogenesis (Feirer *et al.*, 1984), the positive effect of ethylene inhibitors, such as AgNO<sub>3</sub>, on regeneration was thought to be due to enhanced polyamine synthesis rather than reduced ethylene production (Kumar *et al.*, 2009). There have been reports confirming the significance of AgNO<sub>3</sub> on *in vitro* processes in *C. Annuum* (Ashrafuzzaman *et al.*, 2009) and *C. frutescens* (Sharma *et al.*, 2008).

However, no successful report has been documented in Naga chilli so far. As far the *in vitro* fruit formation is concerned, highest number of fruit was achieved in the medium supplemented with GA<sub>3</sub> at a concentration of 3.5mg/L. A total of 6.75 fruits generated from a single explant. BAP concentration was kept constant at 1.5mg/L. GA<sub>3</sub> is known to play important role in shoot elongation and seed germination in many plant species. Apart from this, it is also regarded as a flowering hormone other than ethylene in some plant species. Our investigation complies with that of Buzzy *et al.*, (2005) where the authors suggested possible positive influence of GA<sub>3</sub> on *in vitro* regeneration of Hebenero pepper via organogenesis. In another experiment, Rkhis *et al.*, (2006) reported positive effect of GA<sub>3</sub> on *in vitro* vegetative growth and flowering in olive.

### **Effect of media composition on *in vitro* root induction and acclimatization**

Regenerated plantlets were transferred to root induction medium containing 1.5mg/L of fixed concentration of BAP. IBA was chosen over IAA and NAA as the sole rooting hormone. Alongside, effect of AgNO<sub>3</sub> and GA<sub>3</sub> was also tested. As per figures shown in Table 4, maximum *in vitro* root induction per shoot occurred in T<sub>4</sub> where the medium was fortified with AgNO<sub>3</sub> (3.0mg/L), IBA (3.0mg/L) and GA<sub>3</sub> (2.5mg/L). A highest of 46.65 roots/explant was generated within 14 days after being transferred to rooting medium. Percentage of root induction was also highest with a maximum of 80.96%. However, the longest root of 16.53cm was seen in T<sub>3</sub> (1.5mg/L BAP, 2.5mg/L AgNO<sub>3</sub>, 2.5mg/L IBA and 2.5mg/L GA<sub>3</sub>). Present study reveals positive impact of AgNO<sub>3</sub> on *in vitro* root induction. However, our findings doesn't comply with the work submitted by Anantasaran and Kanchanapoom (2008) where the authors verified that incorporation of AgNO<sub>3</sub> into the medium greatly reduced the rooting percentage in *Zinnia* cultivars. Our investigation is in accordance with the data submitted by Ebida and Hu (1993) where the authors confirmed in their report the supplementation of AgNO<sub>3</sub> into MS medium clearly had some positive effects on regeneration of *in vitro* cultured plantlets of *C. annuum* cv. Early California Wonder. Such variations in results might have occurred due to differences in genotypes and type of explants used. Initially the experiment was conducted with three different forms of auxin, NAA and IAA and IBA. However, in the later stages it was reduced to IBA as the roots cultured in NAA and IAA solutions led to very fragile roots of shorter length. Some earlier work on root initiation prefer IBA over NAA and IAA (Sanatombi and Sharma, 2007; Otrushy *et al.*, 2011; kumari *et al.*, 2012 and Hedgre *et al.*, 2017), while the other preferred

NAA and IAA over IBA (Bodhipadma and Leung, 2003; Siddique and Anis, 2006 and Sanatombi and Sharma, 2008). Another key component to regulate rooting was GA<sub>3</sub>. In the present investigation, we tested the efficacy of GA<sub>3</sub> alongside IBA and AgNO<sub>3</sub>. GA<sub>3</sub> at concentration higher than 2.5- 3mg/L was found to down regulate *in vitro* rooting process. Therefore, it was kept constant at 2.5mg/L. Although, GA<sub>3</sub> is believed to be more involved in shoot and fruit induction processes, our study revealed its positive influence on *in vitro* rooting as well. Number of root induction and percentage of root initiation got drastically reduced upon withdrawal of GA<sub>3</sub> from the medium. As per the experiment conducted by Nanda *et al.*, (1972) root formation on cuttings of *Ipomoea fistula* was stimulated if they were dipped into GA<sub>3</sub> before being transferred to the compost. Rucker (1982) found that GA<sub>3</sub> was able to promote direct root formation in *Digitalis* leaf fragments when applied with low levels of IAA, but became inhibitory when the level of IAA increased. GA<sub>3</sub> when combined with IBA and BAP promoted rhizogenesis from callus of *Pinus taeda* (Tang and Fan, 1999)

*In vitro* regeneration process would not be complete without successful hardening and acclimatization. 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 84.75%. According to work done by Azad *et al.*, (2011), organic farming with the inclusion of biofertilizers in the long-run can be considered an important contributor to food security. Moreover, Ponti *et al.*, (2012), stated that the difference in crop yields under organic and conventional production systems is 20% depending on the crops and regions. Furthermore Bhat *et al.*, (2013) revealed in their investigation that 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. Additionally, it improves soil texture, reduces bulk density and increases the available water content.

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