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Plantlet regeneration of naga king chilli (*Capsicum Chinense* JACQ.) from nodal segments through *in vitro* technique

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Abstract
The present investigation was undertaken at the Department of Horticulture, Nagaland University to standardize the protocol for plantlet regeneration of Naga King Chilli (*Capsicum chinense* Jacq.) by using nodal segments as explants. Nodal segments (1.0 cm) were cultured on MS medium supplemented with either BAP or KIN @ 0-8 mg L^{-1} respectively for shoot proliferation, alone and in combination with 0.5 mg L^{-1} IAA. The medium containing 8 mg L^{-1} BAP + 0.5 mg L^{-1} IAA recorded the maximum response (88.88%) for shoot proliferation. The highest shoots (5.49) with maximum bud length (1.9 cm) were proliferated during the third subculture. Regenerated shoots were maintained in MS basal medium for further elongation thereafter, transferred to MS medium supplemented with auxins viz. IBA and IAA @ 0 - 1.5 mg L^{-1} alone for root formation where 1mg L^{-1} IBA enriched media recorded highest number of functional roots (14.55 per explant). 70% plantlets survival was recorded during hardening.

Keywords: Shoot tip, shooting, regeneration, rooting, auxins

Introduction
Capsicum chinense Jacq. cv. Naga King Chilli is native to North-Eastern India more particularly to Nagaland (Bhagowati and Changkija 2009). It is locally known by various names in different regions such as 'Bhoot jolokia' or 'Bih jolokia' in Assam, 'Naga King Chilli' in Nagaland, 'Umorok' in Manipur and 'Ghostpepper' by the western media. *Capsicum chinense* Jacq. has received the attention of scientific community throughout the world due to its unique aroma and high capsaicin content. Most of the chilli species and varieties cultivated in India contain around 1% capsaicin but Naga King chilli has around 2–4% capsaicin as reported by various researchers (Mathur *et al*., 2000 and Sanatombi and Sharma, 2008) [14, 21].
The Nagaland government had in 1999 passed the Nagaland Geographical Indication of Goods (Registration and Protection) Act, to provide some safety net to Naga farmers in the cultivation of the King Chilli. Nagaland Government has obtained the GI rights for this product in 2008 (Anonymous. 2009) [4].
The conventional method of propagation using seeds is restricted by the short span of viability and low germination rate of chilli seeds. Chilli plants are also highly susceptible to fungal and viral pathogens (Morrison *et al*. 1986) [16]. It is a self-pollinated species but the occurrence of high cross pollination leads to the formation of variants within Naga King Chilli, leading to heterozygosity and true-to-type plants cannot be maintained. Even though other solanaceous members easily undergo morphogenesis, chilli was found to be highly recalcitrant due to the formation of ill-defined bud like structures and rarely of well developed shoots. Since, the plant lacks natural vegetative propagation, plant tissue culture technique provides an alternative method of propagating novel genotypes asexually. Naga King chilli is a species among the chilli group, highly susceptiple to many fungal and viral pathogens. Seeds are recalcitrant, short span of viability and low germination rate. Chilli tissue culture is mostly confined to *Capsicum annum* L. and *Capsicum frutescens* L. and very merge information for *C. chinense* Jacq. Therefore, keeping in mind the problems associated with conventional propagation, the present research involving culture of nodal segment as explants of *Capsicum chinensis* was undertaken to study the effect of different levels of cytokinin (BAP & kinetin) alone and in combination with IAA and levels of auxins (IAA & IBA) in producing shoots and roots and elongation of the shoot buds respectively.

Materials and methods
The present investigation entitled "Plantlet regeneration of Naga King Chilli (*Capsicum chinense* Jacq.) from nodal segments *in vitro* technique" was carried out at the Department of

Horticulture, School of Agricultural Sciences and Rural Development, Nagaland University. The popular genotype *Capsicum chinense Jacq*. cv. Naga King Chili was chosen for the study, because the cultivar is native to North-Eastern India more particularly to Nagaland for its pungency and unique aroma. Healthy and ripe fruits were taken from the selected plants which were grown under proper care at Horticulture Instructional Farm, Nagaland University. Seeds extracted from mature ripe fruits were washed in running tap water and treated with Bavistin solution (0.1%) for 15 minutes followed by rinsing several times with distilled water. The seeds were surface sterilized by immersing in 70% ethyl alcohol for 1 minute under the laminar low cabinet with vigorous shaking, followed by 4% sodium hypochlorite for 5 minutes. The seeds were then rinsed with sterile distilled water to remove the traces of disinfectant liquid and blot dried. Seeds were then sown in petri dishes containing sterile filter paper soaked in distilled water and incubated in dark for 7-10 days at 25±2ºC. After germination, the seeds were inoculated on MS basal medium and allowed to grow. Six-weeks-old *in vitro* germinated seedlings were used as the source of explants. Nodal segments (1.0 cm) were excised from these seedlings and implanted in the culture medium. Murashige and Skoog (1962) medium which has relatively high concentration of nitrate, potassium and ammonium ions when compared to other nutrient media were used in the present investigation.

Nodal segments of 1.0 cm high were cultured in modified MS medium supplemented with ±30gm sucrose in 9 different concentrations of cytokinins (BAP & KIN) @ 0, 2, 4, 6, 8 mg L^{-1} respectively alone and in combination with 0.5 mg L^{-1} IAA, for shoot proliferation. After 4 weeks of multiple buds initiation, Number of shoots responded in each medium, number of buds developed, average length of multiple buds were also recorded. After 4 weeks of culture, best recorded medium was used for further multiplication of propagules, for further regeneration of shoots and growth response at lower concentration of cytokinin in different sub culture treatments. They were subcultured every 3 weeks interval in the best recorded modified MS medium for multiple buds proliferation. The multiple buds were cut separately (containing 1-2 buds each) and transferred into separate bottles containing well-prepared medium for getting enough multiple buds for testing multiplication potentiality in each media. The regenerated shoot buds were placed on MS basal media for shoot elongation. Well elongated shoots were transferred to MS medium supplemented with various concentrations of auxins (IAA & IBA) @ 0, 0.2, 0.5, 0.8, 1.0, 1.2, 1.5 mg L^{-1} alone to optimize protocol for root formation. The rooted plantlets were hardened in pre-sterilized moist cocopeat, sand and farmyard manure @ 1:1:1 ratio and were maintained under shade condition. The effects of treatments were tested by Analysis of Variance; differences among the treatment means were tested by Duncan's Multiple Range Test (DMRT), (Duncan, 1955). The levels of probability used for 'F' test was at 5%.

Table 1: Composition of MS media with different hormone concentrations tested for shoot induction and rooting of Naga King Chilli *(Capsicum chinense Jacq*.)

Medium for shoot induction		Medium for rooting	
Treatment	**Medium concentration (mg L^{-1})**	**Treatment**	**Medium concentration (mg L^{-1})**
M_1S_0	Control	M_1R_0	Control
M_1S_1	2 BAP	M_1R_1	0.2 IAA
M_1S_2	4 BAP	M_1R_2	0.5 IAA
M_1S_3	6 BAP	M_1R_3	0.8 IAA
M_1S_4	8 BAP	M_1R_4	1.0 IAA
M_1S_5	2 BAP + 0.5 IAA	M_1R_5	1.2 IAA
M_1S_6	4 BAP + 0.5 IAA	M_1R_6	1.5 IAA
M_1S_7	6 BAP + 0.5 IAA	M_1R_7	0.2 IBA
M_1S_8	8 BAP + 0.5 IAA	M_1R_8	0.5 IBA
M_1S_9	2 KIN	M_1R_9	0.8 IBA
M_1S_{10}	4 KIN	M_1R_{10}	1 IBA
M_1S_{11}	6 KIN	M_1R_{11}	1.2 IBA
M_1S_{12}	8 KIN	M_1R_{12}	1.5 IBA
M_1S_{13}	2 KIN + 0.5 IAA		
M_1S_{14}	4 KIN + 0.5 IAA		
M_1S_{15}	6 KIN + 0.5 IAA		
M_1S_{16}	8 KIN + 0.5 IAA		

Results and Discussions

Response of different growth regulators to multiple bud induction

The morphogenic responses of the the nodal segments (1.0 cm) were recorded after 6 weeks of inoculation. Maximum response (88.88%) to shoot induction was recorded in M_1S_8, followed by M_1S_7 with 80%. The explants inoculated in controlled medium did not perform any development in respect to shoot proliferation from the nodal segments. The nodal segment explants differed significantly in respect to shoot proliferation under various medium concentrations. The results are in conformity with the findings of Andrzej, G (2002), Hossain *et al*. (2004) and Gururaj *et al*. (2004) [2, 11, 10] in diverse cultivars of *Capsicum annum* L in the regeneration of plants from nodal explants. Treatment difference for the number of shoots per explants was noticed in a time period of 16±1.41 to 35±1.41 days. Shoot bud induction was observed earliest (16.79 days) in M_1S_8 medium and maximum days taken (35.39 days) in M_1S_9 medium (Table 2)

After 6 weeks of inoculation, it was recorded that the maximum number of shoots (4.05) was observed in M_1S_8 which was closely followed by M_1S_7 with the value of 3.33 and M_1S_{16} with 3.25 and found to be at par.

In the present study, the length of the multiple buds did not elongate satisfactorily but the media showed significant differences among them. Among the various media tested, the highest length of multiple buds was recorded in M_1S_8 (1.8 cm).

Plant growth regulators are the essential part of *in vitro* regeneration of crop plants in any artificial medium. Organ

differentiation in plant is regulated by the interplay of auxins and cytokinins (Skoog and Miller, 1957) [24]. By changing the amounts and types of growth regulators in the medium, the cells can be stimulated to develop into shoots and/or roots or may even die, if the medium solidification is changed, the micro-propagation efficiency also altered (Escalona *et al.*, 1999) [7]. Generally, cytokinin helps in shoot proliferation and auxin helps in rooting of proliferated shoots. The regeneration systems of *Capsicum* reported so far had shown the critical effect of cytokinin, cytokinin-cytokinin or cytokinin-auxin ratio in regeneration from various explants (Gunay and Rao 1978, Ezura *et al.* 1993; Anilkumar and Nair, 2004) [9, 3].

This investigation was initiated to study the effect of cytokinins *viz*. BAP and KIN (2-8 mg L^{-1}) alone and in combination with 0.5mg L^{-1} IAA on shoot proliferation from shoot tips and nodal segments of *C. chinense Jacq*. cv. King Chili. The highest mean shoots were obtained on medium supplemented with high levels of BAP alone and in combination with IAA than in KIN. The results are in close conformity with Madhuri and Rajam (1993) who reported that BA was found to be more effective than Kn for multiple shoot proliferation from apical shoot meristem explants of *Capsicum*. The results are constant with those of previous reports in which BAP was used successfully to regenerate shoots (Gunay and Rao, 1978; Hyde and Phillips, 1996; Manoharan *et al.*, 1998 and Ashajyothi, 2004) [9].

Reports show that low concentrations of BAP (8.8-22.2 µM) alone or in combination with 0.6-11.4 µM IAA was found to be effective for shoot bud induction from various explants in chilli tissue cultures (Gunay and Rao, 1978, Agarwal *et al*. 1989, Hussain *et al.* 1999) [9]. However, in the present study, when BAP was used in combination with 0.5 mg L^{-1} IAA, the number of shoot buds increased with the increase in BAP concentration beyond mg L^{-1}. Sanatombi and Sharma (2008) [21] also obtained similar results in *Capsicum chinense* Jacq. cv. Umorok. Similar conclusions that, BAP along with low concentrations of other auxins promoting organogenesis, have been drawn by others (Sanatombi and Sharma. 2007; Gogoi *et al.*, 2014) [20, 8]. This might be attributed to the BAP uptake and metabolism which was subsequently converted to isopentyl adenine (iP) and isopentyl adenosine (ipR) inhibiting biosynthesis activity of cytokinin on cytokinin action of shoot development. The results partially agreed with those of Kumar *et al*. (2010) with respect to the media composition and better induced shoot bud number. They reported that the maximum number of shoot buds per nodal explants was in MS medium supplemented with BAP (5.0 mg L^{-1}) and IAA (0.5 mg L^{-1}) for all the three genotypes of *Capsicum annum L* tested. However, Ahmad *et al.* (2006) [1] and Siddique and Anis (2006) [22] reported that TDZ alone or combined with IAA ranks as the best shoot bud inductor in nodal explants of the cultivar *Capsicum annum* L. cv. Pusa Jwala. Such results obtained may be due to auxins or cytokinins used alone or in combination, which is supposed to be the result of the promotion of biosynthesis or inhibition of degradative metabolism (Singh and Shukla, 2001) [23].

Table 2: Response of different growth regulators to multiple bud induction of nodal segments of *Capsicum chinense Jacq*. cv. Naga King Chilli.

Medium (Treatment)	Number of explants inoculated	Explants responded to multiple shoot formation (%)	Days to shoot initiation	After 6 weeks of culture	
				Number of shoots	Length of shoots (cm)
M_1S_0	20	-	-	-	-
M_1S_1	20	33.33	33.35_{ab}	1.65_{d}	0.9_{c}
M_1S_2	20	44.33	30.45_{b}	1.85_{cd}	1.1_{bc}
M_1S_3	20	46.66	27.15_{bc}	2.45_{c}	1.2_{bc}
M_1S_4	20	63.33	24.68_{cd}	3.10_{b}	1.4_{b}
M_1S_5	20	56.66	23.12_{cd}	2.30_{cd}	1.3_{b}
M_1S_6	20	70.00	20.20_{d}	2.72_{c}	1.5_{ab}
M_1S_7	20	80.00	16.90_{d}	3.33_{bc}	1.6_{ab}
M_1S_8	20	88.88	16.79_{d}	4.05_{a}	1.8_{a}
M_1S_9	20	23.33	35.39_{a}	1.23_{d}	0.5_{d}
M_1S_{10}	20	22.22	33.12_{ab}	1.53_{d}	0.8_{cd}
M_1S_{11}	20	33.33	29.19_{bc}	1.64_{d}	1.0_{bc}
M_1S_{12}	20	44.44	27.72_{bc}	2.68_{bc}	1.1_{bc}
M_1S_{13}	20	44.44	26.04_{c}	1.43_{d}	0.7_{cd}
M_1S_{14}	20	53.33	22.14_{cd}	2.13_{cd}	1.1_{bc}
M_1S_{15}	20	56.66	20.52_{cd}	2.73_{bc}	1.2_{bc}
M_1S_{16}	20	66.66	18.22_{d}	3.25_{b}	1.3_{bc}
SE±			1.41	0.21	0.11
$CD_{0.05}$			4.06	0.59	0.31

Subculturing of *Capsicum chinense* Jacq. cv. Naga King Chili.

Subculture involves the dissociation of the cells from each other and the substrate to generate a single cell suspension that can be quantified. For further regeneration, the medium showing best performance for shoot bud induction was taken forward. In this study the medium M_1S_8 recorded the best response and therefore was taken forward for subculturing studies upto the third cycle. The culture cycle was repeated at three weeks interval. Regeneration with 1-2 nodal segments per explants were subcultured in M_1S_8 medium that remained quiescent for nearly a week. After about 2-3 weeks, explants showed signs of bud growth significantly (Table 3). It was recorded that minimum days (12.25 and 12.58 days) taken for bud initiation was in the 3rd subculture and the maximum (15.42 and 16.55 days) on the 1st cycle respectively. Significant differences in the number of buds per explants were observed at every cycle. The 1st subculture recorded 3.32 numbers of buds and the highest was proliferated during the third subculture (4.37). Maximum length (1.7 cm) of the bud was also recorded on 3rd subculture in case of shoot tip explants. However, maximum numbers of buds (5.49) were recorded at the third subculture cycle with bud length of 1.9 cm. The shortest length of buds was recorded during the first cycle measuring 1.5 cm. It was found that higher concentration of cytokinin and lower levels of auxin is found

beneficial to influence shoot buds formation in the various stages of subculture. It was also observed that explants cultured and subcultured to the same treatment consistently gave lowest production of buds. However, Mok and Norzulaani (2007) [15] reported that bud formation could be enhanced when the explants were subcultured to medium containing same concentration of BAP but reduced concentration of IAA. They also reported that Cytokinin to auxin ratio, their concentration in culture and subculture media, duration of culture and subculture are crucial determinants in the induction of buds and shoot formation. Mok and Norzulaani (2007) [15] also obtained similar trend. However, they recommended subculturing every two weeks in order to obtain higher percentage of bud formation and avoid browning of the explants. Prolonged culture in high concentration of BAP inhibited bud development and leaf expansion, hastened browning and caused abnormality of the explants. Explants without subculture exhibited callus formation and a slower rate of bud induction. The findings are in accordance with the results of Binzel *et al.*, (1996) [5] and Ahmad *et al.* (2006) [1].

The regenerated shoots were separated and replanted for shoot elongation in the basal medium. It was noticed that the regenerated shoots underwent fair shoot elongation of (2.5-4) cm on transfer to growth regulator free MS basal medium. Only vigourous grown shoots after transfer to shoot elongation medium produced elongated shoots. It was observed that among the explants used, the nodal segments gave better elongation after transfer to shoot elongation medium. The results are in accordance with Rahul *et al.* (2015) and Valera-Montero and Phillips (2005) [18, 26].

Although efficient shoot induction has been observed in the present study but transferring of shoot buds in separate medium for further elongation was required. Steinitz *et al.* (1999) reported that shoot bud clusters need to be transferred to separate shoot elongation medium for further elongation which is the major obstacle in obtaining highest length of shoots in pepper. Peddaboina *et al.* (2003) [17] reported that cytokinins commonly stimulate shoot proliferation and inhibit their elongation. The problem of shoot elongation can be overcome by transfer of shoot clusters on secondary medium with lower cytokinin level. The plantlets height was a strong characteristic influenced by the interaction between the increasing regulators and its concentrations. Verma *et al.* (2013) [27] reported that if the shoot obtained after indirect organogenesis was a profuse rosette right from its emergence, its elongation was difficult to achieve but if the shoot emerged bearing well developed leaves, it showed fair elongation in the presence GA_3 in the MS medium.

Table 3: Subculture of *Capsicum chinense* Jacq. cv. King Chilli

Subculture	Explants responded (%)	Days required for multiple bud initiation	After 3 weeks	
			Number of buds	Length of buds (cm)
1st subculture	80.00	16.55	4.33	1.5
2nd subculture	83.33	15.35	4.77	1.7
3rd subculture	86.66	12.58	5.49	1.9
SE±	80.00	0.43	0.12	0.05
$CD_{0.05}$		1.34	0.36	0.15

Effect of growth regulators on rooting

Induction of roots was observed with varying degree of response in all the media tested. Rhizogenesis started within 9-20 days after subculturing into auxin medium, It was clear from the results presented in Table:4 that M_1R_8 was the effective concentration for earlier root initiation recorded 9.41 days followed by M_1R_7 (10.51 days). Maximum time for root initiation 28.11 days occurred at basal medium devoid of growth regulators. The present study investigated after 4 weeks of inoculation recorded that the different levels of IAA and IBA in respect to number of functional roots and length of roots per explants differed significantly. Vigorous mean root length (4.2 cm per explant) was recorded in *in vitro* grown plantlet on MS media supplemented with M_1R_{10} in case of nodal segment used as explants. The effectiveness of IBA on rooting of *in vitro* regenerated *C. chinense* Jacq. plantlets was also reported by Sanatombi and Sharma (2008) [21]. In contrast, this result differed from the study of Kehie *et al.* (2012) [12] who reported that the *C. chinense* plantlets were best rooted in a medium containing IAA with the highest number and maximum length of roots as compared to NAA.

The present observations are consistent with the earlier finding in which IBA was successfully employed for rooting in *Capsicum* Christopher and Rajam (1996) [6], Ahmad *et al.* (2006) [1] and Gogoi *et al.* (2014) [8].

Table 4: Response of different growth regulators on rooting of nodal segments of *Capsicum chinense Jacq.* cv. King Chilli.

Medium (Treatment)	Number of explants inoculated	Explants responded to root induction (%)	Days to root initiation	After 4 weeks of culture	
				Number of roots	Length of roots (cm)
M_1R_0	20	16.66	28.11_{a}	2.32_{f}	1.00_{e}
M_1R_1	20	46.66	11.98_{cd}	6.80_{ef}	2.6_{de}
M_1R_2	20	56.66	14.57_{cd}	7.80_{e}	2.8_{cd}
M_1R_3	20	56.66	17.04_{bc}	9.30_{d}	3.0_{c}
M_1R_4	20	60.00	18.90_{bc}	10.05_{c}	3.8_{ab}
M_1R_5	20	50.00	20.26_{b}	5.40_{f}	2.3_{e}
M_1R_6	20	46.66	20.76_{b}	5.20_{f}	2.0_{e}
M_1R_7	20	63.33	10.51_{d}	9.31_{d}	2.0_{cd}
M_1R_8	20	73.33	9.41_{d}	11.23_{c}	3.4_{a}
M_1R_9	20	80.00	13.82_{cd}	12.75_{b}	3.6_{b}
M_1R_{10}	20	83.33	15.08_{c}	14.55_{a}	4.2_{c}
M_1R_{11}	20	76.66	18.88_{bc}	8.09_{e}	2.5_{d}
M_1R_{12}	20	66.66	19.86_{b}	7.03_{e}	2.2_{d}
SE±			1.28	0.43	0.15
$CD_{0.05}$			3.72	1.24	0.42

Acclimatization.

Plants developed *in vitro* are only partially autotrophic because they are developed in closed, sterile environment and grown on nutrient-rich artificial media under controlled conditions with high humidity and low light intensity. As a result, they have less epicuticular wax and lose water rapidly when transferred to external conditions. Survivability depends on the correct balance between supporting and supply systems.

In the present study, shoots with several well ramified roots were transferred into 10 cm diameter polycups containing pre-sterilized moist cocopeat, sand and farmyard Manure @ 1:1:1 ratio and were maintained under shade condition. This investigation showed that survival of transferred plants improved significantly when plantlets were initially covered with clear polythene bags having a few holes in it and were frequently watered to maintain high humidity and prevent desiccation. After 10 days the humidity was gradually decreased by increasing the size of holes in the polythene bags to harden the plantlets. After 3 weeks, 70% of plantlets survived when covered with polythene bag. The successfully hardened plantlets were indicated by emergence of new apical leaves. The polythene bags were finally removed and the plantlets were ready for transfer to the field condition. The present findings are also in close conformity with several workers. Successful growth and acclimatization of the *in vitro* regenerated plantlets was observed on transplantation to cocopeat mixture and subsequently to 1:1:1 mixture of sand, soil and farm yard manure with 90% survival as reported by Verma *et al*. (2013) [27] and Robinson and Maheswari (2013) recorded about 75% survivability in the same hardening media. Sanatombi and Sharma (2008) [21] also reported successful hardening (90%) of *C. chinense* Jacq. when transplanted to plastic pots containing pre-sterilized sand: soil (5:1) and maintained at 50% shade net house for hardening, by covering the plantlets with perforated polythene bags for the initial 10 days and thereby increased the size of the holes. The plantlets hardened within 15 days with emergence of new apical leaves. The polythene bags were later removed and the plantlets were transferred to pots or field condition. This confirms the earlier observations made by Keyie *et al.* (2012) [12] and Gogoi *et al*. (2014) [8].

Conclusion

Therefore, it would be appropriate and logical suggestion for the conclusion to consider the same protocol and levels of growth regulators 8 mgL^{-1} BAP + 0.5 mgL^{-1} IAA for shoot proliferation and shoot elongation and 1 mgL^{-1} IBA for root induction with modified MS media for refining the technology in respect to *in vitro* regeneration of King chilli as further research studies. This investigation also showed that survival of transferred plantlets improved significantly when plantlets were initially covered with clear polythene bags having a few holes in it than when left uncovered. This efficient and reliable plant regeneration system can be exploited for the production of healthy and disease free plants to enhance yield and productivity through genetic transformation and other cellular techniques.

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