In vitro Induction and Proliferation of Callus in *Piper longum* L. through Leaf Culture

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ABSTRACT

Piper longum L. (Family Piperaceae) is a well-known health promoter used to treat cough, chronic bronchitis, asthma, and diabetes mellitus. The study is aimed to develop a protocol for callus induction and proliferation in *P. longum*. The leaf explants from mature plants were cultured on an MS basal medium supplemented with various concentrations of plant growth hormones, viz. 2,4-Dichlorophenoxyacetic acid (2, 4-D), Kinetin (KN), 6-Benzylaminopurine (BAP), and α-Naphthalene acetic acid (NAA), as well as 10% coconut water. In primary culture, the callus was compact and light white. The best callus induction and growth were observed in the MS basal medium containing1.0 mg/L 2,4-D+ 2.0 mg/L KN at 12 weeks of primary culture. At eight weeks of secondary culture, the MS medium containing 2.0 mg/L BAP alone and 0.5 mg/L NAA + 2.0 mg/L BAP and 10% coconut water had the best callus proliferation. Compared to 2,4-D and KN alone, BAP alone supported rapid callus growth in the MS medium. In *P. longum*, large-scale callus formation from leaf explants could be exploited to produce, isolate, and increase bioactive secondary metabolites for therapeutic purposes.

Keywords: 6-Benzylaminopurine, Growth index, Kinetin, MS media, α-Naphthalene acetic acid, 2,4-Dichlorophenoxyacetic acid

1. INTRODUCTION

Piper longum L. is a valuable medicinal plant, called long pepper in English, Pipla in Nepali, Pipali in Sanskrit, Pippali in Hindi, & Thippili in Srilankan languages. *P. longum* is an endemic plant in the Indo-Malaya region. It is found growing wild in the tropical and subtropical forests of India, Bhutan, Srilanka, Malaysia, Indonesia, Nepal, Singapur, Myanmar, America, etc. In Nepal, it is distributed east to west Terai and Siwalik hills up to 1000 m asl (Press *et al.* 2000). It is a popular medicinal plant in South Asia and the Islamic regions of East and North Africa. Commercially, it is also known as "Pipla" and "Piplamul"; the Pipla is a dried spike (fruit), while a thick root with a stem segment is called Piplamul. The three grades of piplamool are available for use and trade: Grade I with thick roots and underground stems, Grade II and III consisting of thin roots and stems, or broken fragments with a low price compared to grade I (Kumar *et al.* 2011). Dried fruits are also used as seasoning and spices. The alkaloid piperine (3-5%) is the major and active constituent in *P. longum* (Zaveri *et al.* 2010). Fruit (mature female spike), roots (piplamul), leaves, & stem are used for several ailments in the form of juice, decoction, paste, or infusion as a mode of traditional drug preparation, viz. fruits for cough & cold (Muller-Boker 1993; Singh & Maheshwari 1994; Ghimire & Bastakoti 2009), for cough, asthma, & bronchitis (Sigdel & Rokaya 2011; Das *et al.* 2013; Kumar & Bharati 2014; Thapa 2020); stem and leaves for cough (Dangol & Gurung 1991); & roots for cough (Singh 2017), gastritis (Kumar *et al.* 2011), as antitoxin in scorpion sting and snake biting (Chopra *et al.* 1956), and jaundice (Singh *et al.*, 1997). It is also used in stomachic, laxative, anthelmintic, carminative, bronchitis, fever, cold, asthma, urinary discharge, tumors, piles, insomnia, jaundice, leprosy, gout, & rheumatism (IUCN 2004). Plants are decreasing in their natural habitats due to overexploitation for pharmaceutical and traditional medicinal use, habitat destruction, and illegal trade.

The traditional vegetative propagation of *P. longum* is insufficient due to poor seed viability, low percentage of seed germination, and delayed root formation in vegetative cuttings (Sarasan *et al.* 1993). Plant regeneration through seeds is difficult even in vitro conditions in *P. longum*. The callus culture is widely applied in basic research and industrial applications for the regeneration of entire plants and the production of secondary metabolites using precursors and elicitors. Callus and cell cultures have been successfully utilized to produce important pharmaceuticals in relatively large quantities (Pant 2014). Thus, induction of callus using various types of explants in culture is useful for regenerating entire plants through organogenesis or embryogenesis and for producing valuable secondary metabolites. The callus is also used for establishing cell suspensions, as good source material for protoplast isolation, and for extracting pure bioactive chemicals (Jhang *et al.* 1974; Furuya *et al.* 1983).

The callus induction and plant regeneration from leaf and nodal explants in *P. longum* was studied by Sarasan and Nair (1991); Bhat *et al.* (1992); Soniya and Das (2002); Sharon and Maurya (2004); Parida and Dhal (2011); Padhan (2015); Tiwari (2016); Sathelly *et al.* (2016); Fonseka and Wickramaarachchi (2018); Prajapati *et al.* (2019), Wasti and Pant (2019). This paper aims to analyze the callus induction and proliferation in different types of hormones at different concentrations and combinations using leaf explants. It will help to form the basis for the production of secondary metabolites from in vitro raised callus using precursors and elicitors and the phytochemical analysis of callus.

2. MATERIALS AND METHODS

2.1 Plant Material

Plants of *P. longum* were established in pots at the Central Department of Botany, TU, collected from the Rupandehi district, western Nepal, during October-November, 2020. The plant was identified by tallying the herbarium specimen in the Tribhuvan University Central Herbarium (TUCH), Kirtipur (Voucher specimen no. 135 & 136).

2.2 Explants Preparation

The young leaves were carefully excised from the stock plants and washed thoroughly in running tap water, adding a few drops of Tween-20 (Qualigens) for one and a half an hour. It was

again washed four times with distilled water. Then, the explants were surface sterilized with 70% ethyl alcohol for 30-35 seconds and with 0.1 % mercuric chloride solution for 4-5 min. It was washed four times with sterile distilled water to remove the traces of mercuric chloride. Then, leaf discs (0.8–1.0 cm diam.) were prepared with the help of a sterile cork borer in the sterile leaves.

2.3 Culture Medium Preparation

MS (Murashige & Skoog 1962) basal media was prepared from the stock solutions for all the in vitro cultures of leaf explant. For primary culture, the media was added with various concentrations of hormones viz. kinetin (KN) (0.25, 0.5, 1.0, 1.5, 2.0, & 3.0 mg/L) and 2,4-Dichlorophenoxy acetic acid (0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and, 5.0 mg/L) alone as well as in combination, 3% (w/v) sucrose, 10% coconut water (v/v) and 0.8% (w/v) agar. The pH was adjusted to 5.6 before autoclaving. For callus sub-culture, the MS media was supplemented with KN, BAP, & 2,4-D (0.5, 1.0, 1.5, & 2.0 mg/L) alone, and in combination of BAP and NAA (0.5, 1.0, 1.5, & 2.0 mg/L). For control, full, half, and quarter strengths of MS media were prepared without supplementing with the hormones and 10% coconut water. About 25 mL media were dispensed in autoclaved 400 mL sterile jars (78 mm × 122 mm) and closed with aluminium foil. All the media were autoclaved at 121ºC for 20 min at 15 lb/sq. inch.

2.4 Establishment of Cultures

The inoculation of leaf discs was carried out in the laminar airflow chamber. Before the inoculation, the laminar airflow chamber was made sterile by cleaning it with spirit or cotton soaked with 70% ethyl alcohol. The culture tubes and jars containing media, sterile metal instruments, and glassware were exposed to ultraviolet (UV) radiation for 45 minutes to remove the possible contaminants in and around the transfer area. After turning off the UV light, the blower was kept running during the inoculation process. Then, leaf discs (0.8–1.0 cm diam.) were inoculated on the MS medium aseptically. Similarly, the regenerated callus was subcultured on the MS medium after 12 weeks of primary culture. The cultures were maintained at 25 ± 2 °C and 12-16 h photoperiods. Each treatment consists of 3 replicates, and the experiment was repeated three times. Calli of 8 weeks of age were harvested, their growth indices and moisture contents were calculated (Adhikari & Pant 2013) and used for further study.

$$\text{Moisture (\%) of callus} = \frac{\text{Fresh wt of a callus-Dry wt of callus}}{\text{Fresh wt of callus}} \times 100$$

$$\text{Increase (\%)of callus (Growth index)} = \frac{\text{Fresh wt of callus-Fresh wt of callus explant}}{\text{Fresh wt of callus explant}} \times 100$$

3. RESULTS AND DISCUSSION

Callus induction, growth, and proliferation were obtained by inoculating sterile leaf discs of *P. longum* on MS media without addition and supplemented with different concentrations of 2,4-D and KN (0.25, 0.5, 1.0, 1.5, 2.0, & 3.0 mg/L) plus 10% coconut water. All the media did not respond positively to callus induction and growth. All the full, half, and quarter strength of MS basal media without the supplementation of hormones induced callus, but full strength of MS media (45%) induced a large callus than half (30%) and a quarter (25%) strength of MS media. The MS basal media added with various concentrations of kinetin alone did not induce callus. Soniya and Das (2002) reported that MS media supplemented with kinetin alone did not induce callus or direct plant regeneration in leaf explants. However, they induced callus from leaf explant in the MS media supplemented with 2.0 to 5.0 mg/L 2,4-D alone and combined with 2,4-D and KN.

The calli are loosely arranged undifferentiated parenchyma cells arising from the meristematic cells of the parent tissue, mostly from the cambium cells forming over a wounded or cut plant surface. In nature, callus is developed in plants by wounds, tumor-causing bacteria (Ti gene), and genetic tumours (Bhatiya 2015), while it can be produced in vitro culture of a leaf or shoot explants artificially in a suitable nutrient medium. In general, auxin alone and combined auxin and cytokinin induce callus in various plant species. However, callus induction occurs with an intermediate ratio of auxin and cytokinin; root formation occurs with a high ratio of auxin-to-cytokinin, and shoot regeneration occurs with a high ratio of cytokinin-to-auxin (Skoog & Miller 1957). In some species, abscisic acid and brassinosteroid hormones induce callus and might be used instead of auxin or cytokinin for

callus formation (Goren *et al.* 1979; Hu *et al.* 2000). However, auxin and cytokinin are widely studied and used in growth hormones for callus formation, organ regeneration, and metabolite production. The callus can be maintained and preserved for an indefinite period in culture by successive sub-culturing it on the fresh medium.

The best callusing media were MS supplemented with 1.0 mg/L 2,4-D + 2.0 mg/L KN (75%), followed by 0.25 mg/L 2,4-D + 1.0 mg/L KN (65%), 0.25 mg/L2,4-D + 2.0 mg/L KN (65%), 0.5 mg/L 2,4-D + 1.0 mg/L KN (55%), 0.5 mg/L2,4-D + 2.0 mg/L KN (52%), and 1.0 mg/L 2,4-D + 1.0 mg/L KN (45%) at 12 weeks of primary culture. Similarly, MS media added with 2,4-D at higher concentrations (3.0 mg/L to 5.0 mg/L) were found to be in good condition for callus induction (Table: 1). Hussain *et al.* (2011) also induced callus from leaf explant in MS media with a higher concentration of 2,4-D (2.0 mg/L to 3.0 mg/L) in *P. nigrum*. It shows that a low to a high concentration of 2,4-D (2.0 mg/L to 5.0 mg/L) in MS media favors the callus induction in *P. longum*. Similarly, by increasing the concentration of 2,4-D from 0.25 mg/L to 1.0 mg/L at the constant 1.0 mg/L to 2.0 mg/L KN in MS media, the induction and proliferation of callus were increased and then decreased. All the calli were compact and light white in primary culture. The callus may be friable or compact and may or may not contain somatic embryos (embryonic callus), roots (rooty callus), and shoots (shooty callus), which depends on the explants, plant hormones, culture condition, and plant species. The earlier workers found that the best condition for callus induction and growth from leaf segment was MS media added with 1.0 mg/L IAA + 1.0 mg/L BAP (Sathelly *et al.* 2016); with 0.5 mg/L TDZ (Prajapati *et al.* 2019); with 1.0 mg/L 2,4-D + 1.5 mg/L BAP (Malthi *et al.* 2016); with 1.0-2.0 mg/L 2,4-D + 1.0 mg/L BA (Sarasan *et al.* 1993) in *Piper longum*. Similarly, the best condition for callus induction and growth from leaf segment was MS media supplemented with 3.0 mg/L NAA + 0.05 mg/L BAP in *Piper betle* (Johri *et al.*1996); with 0.5 or 1.5 mg/L BA + 1.0 mg/L NAA in *Piper nigrum* (Ahmad *et al.* 2010); with 1.0 mg/L BA + 0.5 mg/L GA3 in *Piper nigrum* (Ahmad *et al.* 2013); with 2.0 mg/L 2,4-D + 1.5 mg/L KN in *Piper auritum* (Dominguez 2006); with 1.0 mg/L NAA + 1.0 mg/L BAP in *Bergenia ciliata* (Shrestha & Pant 2011); with 1.5 mg/L NAA + 10.0 µM SNP (Sodium Nitroprusside) + 10% coconut water in *Valeriana jatamansi* (Pandey *et al.* 2020); with 1.0 mg/L 2,4-D + 0.5 mg/L BAP in *Sonchus arvensis* (Wahyuni *et al.* 2020). It shows that the callus induction occurs from the leaf explants in MS media added with various hormones, viz. IAA, BAP, BA, NAA, TDZ, 2,4-D, and GA3 alone and in combination. Callus induction within a plant species depends on the type of explants (leaf, stem, or rhizome), the orientation of the explants, growth hormones, maturity of the plant and explants (juvenile or old), medium composition, metabolic condition of the plant, temperature, the growth conditions, and donor plant variety (Klimek-Chodacka *et al.* 2020). However, the plant species depends on successful callus induction, growth, and proliferation.

Table. 1 Callus induction in various concentrations of 2,4-D and Kinetin.

2,4-D/→ KN↓	**0.0 mg/L**	**0.25 mg/L**	**0.5 mg/L**	**1 mg/L**	**1.5 mg/L**	**2 mg/L**	**3 mg/L**	**4 mg/L**	**5 mg/L**
0.0 mg/L	LC	-	-	-	-	LC	MC	HC	HC
0.25 mg/L	-	-	-	-	LC	LC	LC	MC	LC
0.5 mg/L	-	-	MC	MC	MC	MC	MC	LC	LC
1.0 mg/L	-	HC	HC	HC	MC	LC	-	-	-
1.5 mg/L	-	MC	MC	MC	MC	LC	-	-	-
2.0 mg/L	-	HC	HC	HC	MC	LC	-	-	-
3.0 mg/L	-	HC	HC	MC	MC	LC	-	-	-

MS media, Culture condition: 25 ± 2 °C, 12-16 h Photoperiods, 12 weeks

*LC=Low callus (<1.0 g fresh wt.), *MC=Medium callus (1.0-2.0 g fresh wt.), *HC=High callus (>2.0 g fresh wt.)

The induced calli from 1.0 mg/L 2,4-D + 2.0 mg/L KN were maintained by subculturing in the same concentration of 2,4-D and KN in MS media, and then the calli were subcultured in MS basal media added with different concentrations of 2,4-D, Kinetin and BAP alone, as well as in the combination of NAA and BAP, and 10% coconut water. The proliferation of callus was found in all concentrations. However, the best callus proliferation was found in MS basal media supplemented with 2.0 mg/L BAP alone, which was determined based on the fresh weight and dry weight of calli after eight weeks of subculture (Table 2). At this concentration, 2.149±0.521 g fresh weight callus was obtained from the 0.219 ± 0.030 g initial fresh weight of callus explant, which was a 674 % increase of callus growth after eight weeks of subculture. After the subculture, callus morphology changed from compact light white to slightly friable yellow, compact yellow, and compact green. Calli in MS media added with auxin (2,4-D) were slightly friable yellow, but calli in MS media supplemented with cytokinins (KN and BAP) were compact green and compact yellow. Change in callus morphology after subculture might be due to genetic heterogeneity in callus and hormonal composition in media. In tissue culture, explants with different genotypes show different responses under similar growing conditions (Nehara *et al.* 1989 & 1990). Plant hormones affect cell growth, organogenesis, and metabolite synthesis (Lian *et al.* 1991). The proliferation of callus reduced, decreasing the concentration of all hormones, viz. 2,4-D, KN, and BAP alone in MS basal media.

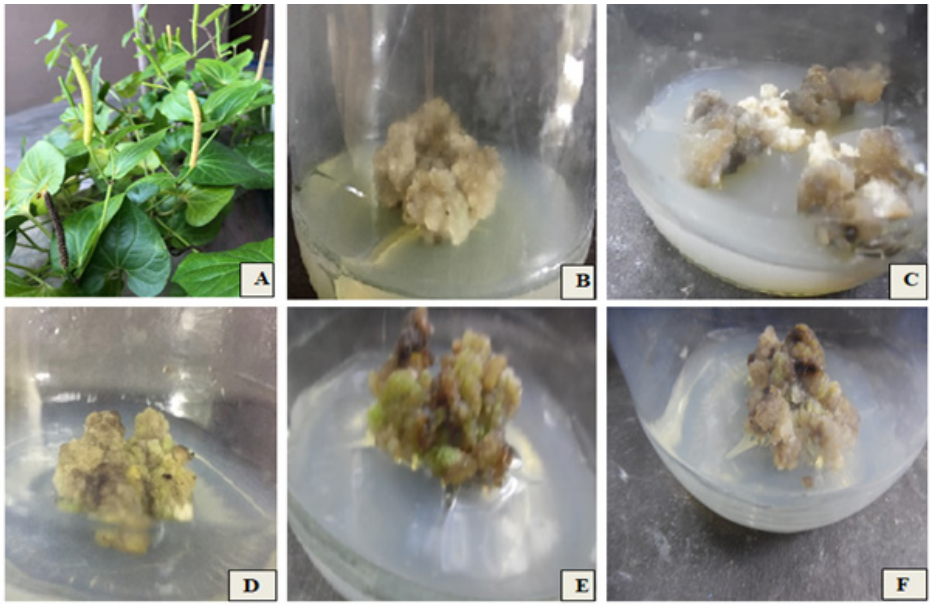

Fig. 1 (A): *Piper longum* plant; (B): Callus: MS + 1.0 mg/L2,4-D + 2.0 mg/L KN; (C): MS + 0.25 mg/L 2,4-D + 1.0 mg/L KN; (D): MS + 0.5 mg/L 2,4-D + 1.0 mg/L KN; (E): MS + 0.5 mg/L2,4-D + 2.0 mg/L KN; (F): MS + 1.0 mg/L 2,4-D + 1.0 mg/L KN

Cytokinins (Kinetin and BAP) alone were more effective than auxins (2,4-D) for callus proliferation in MS media in *P. longum*. The p-value from two way ANOVA test of callus growth (g) versus plant growth hormones (p-value<0.05, i.e.=0.0116) and various concentrations of 2,4-D, KN, and BAP (p-value<0.05, i.e.= 0.0289) showed significant difference It shows that the callus proliferation depends on types of plant hormones and concentrations supplemented in MS media. The callus proliferation increases by increasing the concentration of 2,4-D alone, KN alone, and BAP alone from 0.5 mg/L to 2.0 mg/L in MS media.

Table: 2 Individual effects of 2, 4-D, KN, & BAP on the growth and development of callus after secondary culture (observation at eight weeks)

S. N.	2,4-D (mg/L)	KN(mg/L)	BAP(mg/L)	Fresh wt (g) of callus explant	Fresh wt (g) of callus	Dry wt (g) of callus	Increase (%) of callus
1	0.5			0.220±0.029 CW	0.401±0.015 FLY	0.102±0.013	182
2	1			0.232±0.070 CW	0.544±0.057 FLY	0.122±0.020	234
3	1.5			0.235±0.073 CW	0.571±0.025 FLY	0.129±0.015	243
4	2			0.227±0.078 CW	0.950±0.093 FLY	0.213±0.047	419
5		0.5		0.222±0.076 CW	1.006±0.082 CY	0.262±0.059	312
6		1		0.213±0.033 CW	1.223±0.330 CG	0.227±0.013	359
7		1.5		0.224±0.029 CW	1.141±0.071 CG	0.189±0.012	352
8		2		0.209±0.053 CW	1.677±0.021 CG	0.317±0.019	543
9			0.5	0.236±0.030 CW	0.702±0.090 CY	0.139±0.028	209
10			1	0.226±0.077 CW	1.057±0.113 CG	0.204±0.013	324
11			1.5	0.257±0.041 CW	1.751±0.262 CG	0.346±0.048	430
12			2	0.219±0.030 CW	2.149±0.521 CG	0.367±0.106	674

MS media, Culture condition: 25 ± 2 °C, 12-16 h Photoperiods, eight weeks

*CW: compact light white, CG: compact green, CY: compact yellow, FLY: friable light yellow. Data were obtained from a total of 36 explants with repeated three experiments.

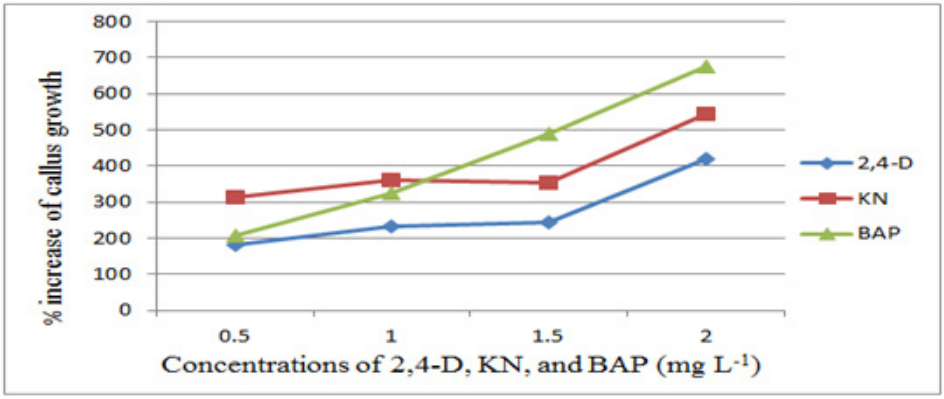

Fig. 2 Callus proliferation in MS media added with different concentrations of 2,4-D, KN, and BAP

Similarly, the best callus proliferation was obtained in MS media added with 0.5 mg/L NAA + 2.0 mg/L BAP in terms of fresh weight and dry weight after eight weeks of subculture (Table 3). At this concentration, 1.843±0.134 g fresh weight of callus was obtained from the 0.316 ± 0.011 g initial fresh weight of callus explant after eight weeks of subculture. All the calli after subculture were changed in morphology from compact light white to compact white brown, compact yellow, compact green, and slightly friable light yellow, which may be due to the release of secondary metabolites, change in hormonal composition in MS media, or vitrification. At a constant concentration of BAP (cytokinin), increasing the concentration of NAA (auxin) from 0.5 mg/L to 2.0 mg/L in MS media resulted in a decrease in callus growth and development. It shows that the higher concentration of auxins in combination with cytokinins does not favor the callus growth and proliferation of *P. longum*.

Table: 3 Combined effect of NAA + BAP on the growth and development of callus following secondary culture (observation at eight weeks)

S. N.	NAA (mg/L)	BAP (mg/L)	Fresh wt (g) of callus explants	Fresh wt (g) of callus	Dry wt (g) of callus	Increase (%) of callus
1	0.5	0.5	0.379±0.047 CW	2.089±0.185 CWB	0.541±0.067	551
2	1	0.5	0.340±0.042 CW	1.732±0.266 CG	0.431±0.078	509
3	1.5	0.5	0.309±0.055 CW	0.856±0.079 CY	0.206±0.028	277
4	2	0.5	0.363±0.061 CW	0.727±0.045 CY	0.171±0.008	200
5	0.5	1	0.331±0.037 CW	1.642±0.150 CG	0.426±0.049	496
6	1	1	0.394±0.014 CW	1.178±0.052 CG	0.290±0.039	298
7	1.5	1	0.338±0.023 CW	0.716±0.176 CG	0.162±0.027	211
8	2	1	0.386±0.015 CW	0.630±0.071 FLY	0.156±0.023	172
9	0.5	1.5	0.344±0.007 CW	1.465±0.098 CG	0.361±0.045	425
10	1	1.5	0.314±0.017 CW	1.132±0.147 CY	0.258±0.011	360
11	1.5	1.5	0.308±0.020 CW	0.744±0.096 FLY	0.183±0.029	241
12	2	1.5	0.387±0.014 CW	0.628±0.120 FLY	0.157±0.040	162
13	0.5	2	0.316±0.011 CW	1.843±0.134 CWB	0.428±0.004	583
14	1	2	0.363±0.011 CW	1.561±0.064 CY	0.390±0.051	430
15	1.5	2	0.397±0.025 CW	0.644±0.131 CY	0.158±0.042	162
16	2	2	0.344±0.045 CW	0.543±0.104 CG	0.146±0.031	157

MS media, Culture condition: 25 ± 2 °C, 12-16 h Photoperiods, eight weeks

*CW: compact light white, CG: compact green, CY: compact yellow, FLY: friable light yellow, CWB: compact white brown. Data were obtained from a total of 36 explants with repeated three experiments.

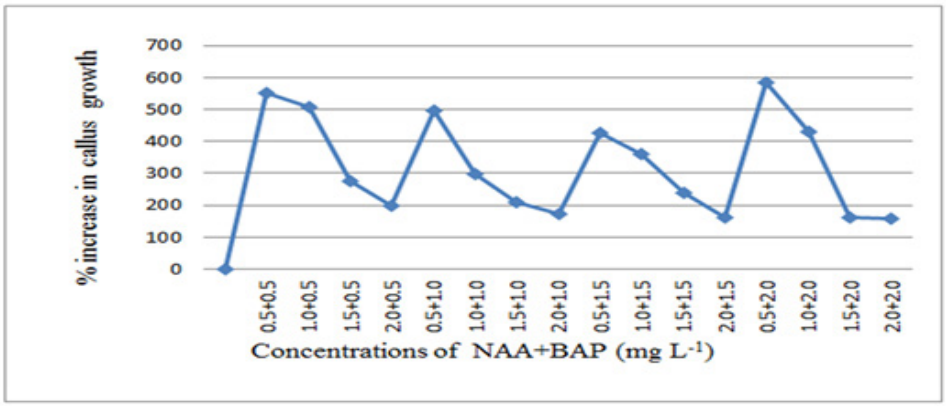

Fig: 3 Callus proliferation in MS media supplemented with various concentrations of NAA + BAP

Using an organic additive (10% coconut water) in MS media was effective for the induction and proliferation of calli in the leaf explant of *P. longum*. The callus induction in control was less than the supplemented hormones, viz. 45% (full MS), 30% (half MS), & 25% (quarter MS) less compared to MS media supplemented with 10% coconut water and plant hormones. Gnasekaran *et al.* (2010) argued that coconut water acts as a cytokinin-like growth regulator containing many nutritional and hormonal substances that induce cell division and growth in culture.

3. CONCLUSION

Callus can be induced from the leaf segments in MS media containing 2,4-D alone and in the combination of 2,4-D and KN. The callus induction in *P. longum* was favored by a high concentration of 2,4-D (auxin) alone and a comparatively low concentration of 2,4-D with a high concentration of KN (cytokinin) in conjunction with MS media. Increased concentrations of 2,4-D alone, BAP alone, and KN alone in MS media increased callus growth and proliferation. However, no definite callus growth and proliferation pattern was observed in MS media in combination with NAA and BAP at secondary callus culture. The protocol developed for the in vitro induction, growth, and proliferation of callus from leaf segment in *P. longum* could be useful for producing and enhancing valuable secondary bioactive compounds using elicitors. Plant regeneration in *P. longum* can also be accomplished from the callus via organogenesis and somatic embryogenesis, and it is in progress.

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