Asian Journal of Biology

8(3): 1-8, 2019; Article no.AJOB.53376
ISSN: 2456-7124

Effect of Chitooligosaccharide on *In vitro* and *Ex vitro* Growth of *Piper nigrum* L.

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Authors' contributions

This work was carried out in collaboration among all authors. Author MQQ wrote the protocol, designed the study and performed the statistical analysis. Authors NTD and NQV wrote the first draft of the manuscript, managed the analyses of the study and managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJOB/2019/v8i330066
Editor(s):
(1) Dr. P. Dhasarathan, Department of Biotechnology, Prathyusha Engineering College, Anna University, India.
Reviewers:
(1) Yahaya Kudush Kawa, Njala University, Sierra Leone and Horticultural Research Institute, Agricultural Research Center (A.R.C.), Egypt.
(2) Vipul Kumar, Lovely Professional University, India.
(3) Kiran Timilsina, Agriculture and Forestry University, Nepal.
Complete Peer review History: http://www.sdiarticle4.com/review-history/53376

Original Research Article

Received 17 October 2019
Accepted 21 December 2019
Published 31 December 2019

ABSTRACT

Oligosaccharins: oligogalacturonic, and chitooligosaccharides are known as molecular signals to induce and regulate various genes in plants. This study was conducted to deternine the effects of chitooligosaccharide on bud formula and growth of *Piper nigrum* in both *in vitro and ex vitro.* The results showed that sterilize *Piper nigrum* shoots with 30% sodium hypochlorite at 10 min was the most suitable condition; appropriate culture media for bud formulation was Murashige and Skoog (MS) media supplemented 30 g/L saccharose, 7,5 g/L agar, 3 mg/L N^6 – benzyl adenine (BA), culture media for growth of plantlet shoot was MS media supplemented 30 g/L sacharose, 7,5 g/L agar, 1 g/L NAA, 2 mg/L IBA and 45 ppm chitooligosaccharide. Supplementation of chitooligosaccharide at concentration of 45 ppm was optimal for the growth of *Piper nigrum* plantlets both *in vitro* and *ex vitro.* Present study indicated that chitooligosaccharide strongly promote the growth of *Piper nigrum* and recommend concentration for both *in vitro* and *ex vitro* is 45 ppm.

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Keywords: Piper nigrum L.; chitooligosaccharide; chitosan; plant tissue culture.

1. INTRODUCTION

Auxin, gibberellin, cytokinin, ethylene and absicic are commonlywell-known as plant growth regulators. Recently, oligosaccharins have been found as the novel plant growth regulators [1]. Molecular signals of numerous genes in plants were induced and regulated by several oligosaccharins such as chitooligosaccharides, oligogalacturonic and N-acetyl oligoglucosamine. Chitosan and chitooligosaccharides are also known as molecular signals to induce plant promoter and develop disease resistance system in plants [2]. Chitooligosaccharides can activate more than 20 pathogenesis-related genes such as ARnase, chitinases, β-glucanase, phytoalexins, lignin, and genes related to metabolisms of plants [3,2]. Plant growth promoter and antifungal activity of chitosan and their derivatives have been demonstrated on many plants in the field such vegetables [4], maize [5], soy bean [6,7], peanut [8], rice, black pepper, cotton [9], coffee [10,11] and flowers [12]. Chitosan and its derivatives also increase chlorophyll content, size of chloroplast and reduction of transpiration of the leaves of plants soy bean [7], peanut [8], coffee, black pepper [13,2] and orchid [9].

Chitosan and chitooligosaccharides were widely used for plant tissue culture *in vitro*. Study on effects of chitosan glutamate on growth of carrot (*Daucus carota* L) plantlets indicated that supplementation of 100 mg chitosan glutamate/l to MS medium resulted in high conversion of somatic embryo- derived plantlets. Using irradiated chitosan at 100 kGy in carrot, strawberry and *L. latifolium* increased fresh biomass by 37%, 36% and 32,3% respectively [14,15,16]. In addition, molecular and DD% of chitosan affected on plant growth level, chitosan irradiated at 100 kGy with a Mw of 16 kDa inhibited the strongest effective on the growth of plants *in vitro* [16]. Moreover, optimal supplemented concentration of chitosan for each plant was various. For example, suitable chitosan concentration for growth of shoot clusters and shoot multiplication rate rate was 70 ppm for *Chrysanthemum*, 50~100 ppm for *Lisianthus* and 30~100 ppm for *Limonium* [15]. Chitosan was also used as a growth promoter for tissue culture of orchid *Dendrobium phalaenopsis* [17]. Results of this study showed that low molecular weight of chitooligosaccharide (less than 1 kDa) was more effective on growth of protocorm-like bodies of the orchid than big molecular weight (10 kDa and 100 kDa), and the optimal concentration was 15 ppm [17]. Comparison of effectiveness of plant growth stimulation of chitosan from shrimp and fungi, fungal chitosan of 10 kDa was the most effective in enhancing fresh weight of protocorm and number of plantlets generated by orchid protocorm [17,18]. Application of chitooligosaccharides for tissue culture of black pepper *in vitro* has not been any papers published yet.

Black pepper has long been considered a significant daily spice, amounting to 20% of all world spice imports [19]. Black pepper has been planted widely in Vietnam, Indonesia, India, and Brazil. Of these, Vietnam is the largest producer and exporter of peppercorns with the total 546,000 tons, taking about 40% of the world black pepper production. In Vietnam, black pepper trees have been mainly cultivated in the Central Highlands [20]. However, lack of virus clean black pepper nursery supply has been affecting the production of this area. The aim of this research was to determine suitable concentration of auxin, kinetin and chitooligosaccharide for growth of root and shoot of black pepper in tissue culture (*in vitro)* and in green house (*ex vitro)*.

2. MATERIALS AND METHODS

2.1 Materials

Chitooligosaccharide with Mw: 20 kDa, DD 85% was a product of Keumho Chemical Co., Ltd, Korea. Piper nigrum shoots were collected from WASI (Western Highland Agriculture and Forestry Science Institute). Napthyl acetic acid (NAA), Indol butyric acid (IBA), N^6 – benzyl adenine (BA) and kinetin were purchased from Merck (Germany).

2.2 Culture Medium

Medium of tissue culture of *Piper nigrum* in vitro was MS (Mugashige and Skoog, 1962) supplmented with vitamin Morel, saccharose 30 g/L, agar 7 g/L, adjusted to pH 5.8.

2.3 Experiments

Shoot tip explants of black pepper (*Piper nigrum*) were sterilized with sodium hypochlorite. Concentrations of sodium hypochlorite used to

Table 1. Effect of concentration of sodium hypochloride and time of treatment on efficiency of sterilization of *Piper nigrum* tissue

Formulas	Sodium hypochlorite concentration % (v/v)	Time (min)
$S_{10}T_{10}$	10	10
$S_{10}T_{30}$	10	30
$S_{20}T_{10}$	20	10
$S_{20}T_{30}$	20	30
$S_{30}T_{10}$	30	10
$S_{30}T_{30}$	30	30

Table 2. Effect of chitooligosaccharide on the growth of *Piper nigrum in vitro*

Formulas	NAA(mg/L)	IBA(mg/L)	Chitooligosaccharide (mg/L)
C_0	1	2	0
C_{15}	1	2	15
C_{30}	1	2	30
C_{45}	1	2	45
C_{60}	1	2	60

be 10, 20, 30% (v/v) and time of treatment to be 10 and 30 minutes as follows.

Disinfected shoots were placed in tubes with MS medium (Murashige and Skoog 1962) added with vitamin Morel, saccharose 30 g/l, agar 7 g/l, adjusted to pH 5.8. MS medium for tissue culture *in vitro* of *Piper nigrum* was supplemented 0; 1; 2; 3 mg/l BA; and 1 mg/L NAA.

MS medium for tissue culture of *Piper nigrum* was supplemented IBA, NAA and chitooligosaccharide as follows.

Effect of chitooligosaccharide on the growth of *Piper nigrum* in green house: *Piper nigrum* plants in flasks were transferred in the green-house for a 30 days-long acclimatization period. Then plantlets with 3 entire leaves were planted in sandy soil mixing with straw and coconut coir (1:1:1). Plants were distributed in the green house using a randomized complete block experimental design with five plots of thirty plants each. Nursery plants were sprayed Grown more (N-P-K: 30-10-10) at concentration of 1 g/L, 500 ml every week, and irrigated water one time per day. Each plan plot was sprayed with 1 litre of chitooligosaccharide at concentration of 0, 15, 30, 45 or 60 ppm each 15 d from day 15^{th} to 60^{th}.

2.4 Condition for *In vitro* Cultivation

The plant tissue and plantlets were cultivated in Erlenmeyer flash 250 ml, light intensity of 35 µmol $m^{-2}s^{-1}$ for 12 h per day; 25±2°C and 60 ± 5% humidity.

2.5 Condition for Greenhouse

Nursery plants were grown in control conditions, sun light was reduced to 50%, temperature 25-34°C, average humidity 70% ± 10%.

2.6 Statistical Analysis

Statistical analysis was performed using analysis of variance (ANOVA) and followed by Duncan's multiple range test with triplicate by MSTATC software, P-value ≤ 0.05 considered as significant.

3. RESULTS AND DISCUSION

3.1 Effect of Concentration of Sodium Hypochlorite and Time of Treatment on Efficacy of Sterilization

Sodium hypochlorite with different concentrations (10 % to 30%) and treatment times (10 minutes to 30 minutes) were used to sterilize pepper shoots. The results showed that sodium hypochlorite concentration affected on the contamination rates and live rates of black pepper shoots, increase sodium hypochlorite concentration significantly reduced contamination rate (Table 3). However, treatment with high dose of sodium hypochlorite (30% v/v) caused shoots death, particularly with long treatment time (30 minutes). The results indicated that the most suitable sterilization condition for black pepper shoot was 30% sodium hypochlorite at 10 minutes (Table 3). Previous studies have reported that sodium hypochlorite is an effective

method for sterilization and each plant/plant part has different suitable concentration and time of treatment. For example, sodium hypochlorite at 5.25 % was the most effective for sterilization of *dendrobium* orchid, while that for sweet potato was 20% for 30 minutes [21].

3.2 Effect of BA on Bud Formation and the Growth of Shoots of *Piper nigrum* after 30 d *In vitro*

BA was significant effective on bud formation, number of bud, shoot height and number of leaf (Table 4). At the highest concentration of BA (3 mg/L) using in this study was found to be the most suitable for bud formation and shoot growth as the number of bud, shoot height and number of leaf were the highest among 4 concentrations.

3.3 Effect of IBA and NAA on the Growth of Shoot and Root of *Piper nigrum In vitro* after 45 Days

IBA and NAA at 4 different concentrations were used for investigating effects of plant regulators on the growth of shoot and root of the plantlets after 45 days cultivation. Different concentration of both IBA and NAA significant affected on number of root, length of root, fresh biomass of root, number of leaf, height of shoot and fresh biomass of shoot (Table 5). MS Media supplement with 1 mg/L NAA and 2mg/L IBA showed the most effective on growth of *Piper nigrum* shoot in vitro, all of the growth parameters were highest at this concentration (Table 5). Auxins such as IBA and NAA effect on growth of plant both *in vitro* and *ex vitro*, and optimal concentration of these plant regulators for plant growth is various in each plant. The suitable concentration of IBA and NAA for *Piper nigrum* shoot growth were higher when compared with other plants such as sweet potato, *Chrysamthemum* and *Lisianthus*; MS media supplemented with 1 mg/L and 0 mg/L IBA and NAA was suitable for sweet potato [21] while that for *Chrysamthemum* and *Lisianthus* were 0.1 mg/L, 0.3 mg/L IBA and 1 mg/L, 0.01 mg/L, respectively [15,16].

3.4 Effect of Chitooligosaccharide on the Shoot Growth of *Piper nigrum* Plantlets *In vitro* Cultivation

MS supplemented with 0, 15, 30, 45, 60 mg/L chitooligosaccharide were significantly influenced on shoot growth of *Piper nigrum* plantlets *in vitro* cultivation (Tables 6, 7). Increase of chitooligosaccharide concentration from 0-45 mg/L resulted in increasing number of leaf, fresh weight of shoot and dry weight of shoot after 30 days and 45 days of cultivation (Tables 6, 7). However, in higher concentration (60 mg/L) the fresh weight of shoot, dry weight shoot and height of shoot were reduced. The shoot growth was highest at 30 mg/L chitooligosaccharide, but at this concentration the number of leaf was less than at 45 mg/L and 60 mg/L chitooligosaccharide. Both fresh weight of shoot and dry weight of shoot showed the highest at the concentration of 45 mg/L and at this concentration of chitooligosaccharide plantlets grew stronger than in other concentrations (Fig. 1).

Table 3. Effect of concentration of sodium hypochlorite and treatment time on efficacy of sterilization

Formulas	Contamination rate				
	Bacteria %	Fungi %	Total %	Died and no contamination	Alive and no contamination
$S_{10}T_{10}$	13.33	86.67	100	0^{by}	0^{e}
$S_{10}T_{30}$	3.33	90	93.33	0^{b}	6.67^{e}
$S_{20}T_{10}$	16.67	66.67	83.33	0^{b}	16.67^{d}
$S_{20}T_{30}$	3.33	70	73.33	0^{b}	26.67^{c}
$S_{30}T_{10}$	3.33	16.67	20	6.67^{b}	73.33^{a}
$S_{30}T_{30}$	0	3.33	3.34	33.33^{a}	63.33^{b}
ANOVA[z]					
Concentration	*	**	*	**	**
Time	**	ns	**	**	ns
Concentration and time	ns	ns	ns	**	*
CV (%)	29.06	11.22	39.08	50	16.94

*Where: **, *, significant differences at P 0.01, 0.05 and ns: no significant differences*
Note: Different superscripts in the same column are significantly different between the treatments at 5% level according to Duncan's Multiple Range Test

Table 4. Effect of BA on bud formation and the growth of shoot of *Piper nigrum* after 30 days *In vitro*

Formulas	% bud formation	Number of bud	Height of shoot (mm)	Number of leaf
BA_0	100	0,56[d]	1.59[d]	0,89[c]
BA_1	100	1,78[c]	5.67[c]	3,33[c]
BA_2	100	3,34[b]	8.78[b]	7,56[b]
BA_3	100	5,22[a]	14.22[a]	11,11[a]
ANOVA	-	**	**	**
CV (%)	-	7,13	18.54	12,82

*Where: **, *, significant differences at P 0.01, 0.05 and NS: no significant differences*
Note: Different superscripts in the same column are significantly different between the treatments at 5% level according to Duncan's Multiple Range Test

Table 5. Effect of IBA and NAA on the growth of shoot and root of *Piper nigrum* in vitro after 45 days

Formulas	% root formation	Number of root	Length of root (cm)	Fresh biomass of root (g)	Number of leaf	Height of shoot (cm)	Fresh biomass of shoot (g)
NAA_1IBA_0	100	1.22[c]	1.18[c]	0.0019[d]	3	3.03[c]	0.0972[d]
NAA_1IBA_1	100	3.89[b]	2.01[b]	0.0318[b]	3.78	5.28[a]	0.3054[b]
NAA_1IBA_2	100	4.25[a]	2.78[a]	0.0521[a]	3.22	4.42[b]	0.4024[a]
NAA_1IBA_3	100	1.89[c]	1.02[c]	0.0105[c]	3.56	1.79[d]	0.1176[c]
ANOVA	-	**	**	**	ns	**	**
CV (%)	-	7.27	5.23	1.02	6.19	1.57	6.24

*Where: **, *, significant differences at P 0.01, 0.05 and NS: no significant differences*
Note: Different superscripts in the same column are significantly different between the treatments at 5% level according to Duncan's Multiple Range Test

Table 6. Effect of chitooligosaccharide on the shoot growth of *Piper nigrum* plantlets *In vitro* cultivation after 30 days

Formulas	Number of leaf	Height of shoot (cm)	Fresh weight of shoot (g)	Dry weight of shoot (g)
C_0	3.18[d]	4.30[d]	0.384[e]	0.042[e]
C_{15}	4.14[c]	5.00[c]	0.459[d]	0.057[d]
C_{30}	4.71[b]	7.24[a]	0.611[c]	0.076[c]
C_{45}	5.77[a]	6.27[b]	1.076[a]	0.143[a]
C_{60}	5.48[a]	5.03[c]	0.802[b]	0.114[b]

Note: Different superscripts in the same column are significantly different between the treatments at 5% level according to Duncan's Multiple Range Test

Table 7. Effect of chitooligosaccharide on the root growth of *Piper nigrum* plantlets after 45 days *In vitro*

Formulas	Number of root	Length of root (cm)	Fresh weight of root (g)	Dry weight of root (g)
C_0	4.23[d]	2.71[c]	0.0317[e]	0.004[e]
C_{15}	5.48[b]	2.29[c]	0.0443[d]	0.006[d]
C_{30}	5.82[a]	3.48[b]	0.0873[b]	0.012[b]
C_{45}	5.41[bc]	8.11[a]	0.2037[a]	0.029[a]
C_{60}	5.13[c]	2.79[bc]	0.0663[c]	0.009[c]

Note: Different superscripts in the same column are significantly different between the treatments at 5% level according to Duncan's Multiple Range Test

The results in this study was consistent with previous studies on the effect chitooligosaccharide on growth of plant *in vitro*. Nge et al. [17] studied effect of chitooligosaccharide on *Dendrobium* orchid and reported that 20 mg/L chitooligosaccharide was

most effective to shoot and root growth, and chitooligosaccharide with molecular weight of 1kDa was more effective than 10 and 100 kDa [17]. In other study, the results indicated that the gamma irradiated chitosan was active on the growth of *Chrysanthenum* and *Lisianthus in vitro,* the optimal concentration of chitosan for these plants were 50 ppm and 30 ppm, respectively [16].

3.5 Effect of Chitooligosaccharide on the Shoot Growth of *Piper nigrum* Plantlets *Ex vitro*

Each plan plot was sprayed with 1 liter of chitooligosaccharide at concentration of 0, 15, 30, 45 or 60 mg/L each 15 day from day 15th to 60th. The results have showed that application of chitooligosaccharide significant increased the shoot and root growth in compared to the control (0 mg/L) (Tables 8, 9). 45 mg/L chitooligosaccharide concentration was the most effective to the shoot growth while 60 mg/L was the most effective to the growth of root. These results also consistent with previous studies on the effects of chitosan on plant growth. In other studies, Dzung and Thang, Dzung and Thuoc [7,8,22] also reported that chitooligosaccharide enhanced growth of soybean, peanut and rice on field condition, optimal chitooligosaccharide concentration for growth of these plants were ranged from 30-50 mg/L.

Fig. 1. Effect of chitooligosaccharide on the root growth of *Piper nigrum* plantlets after 45 days *in vitro*

Note: Bar indicates 1 cm

Table 8. Effect of chitooligosaccharide on the shoot growth of *Piper nigrum* plantlets after 70 days *Ex vitro*

Formulas	Number of leaf	Height of shoot (cm)	Fresh weight of shoot (g)	Dry weight of shoot (g)
CE_0	5.12^{c}	19.27^{e}	4.493^{c}	0.405^{c}
CE_{15}	8.84^{a}	22.50^{c}	9.167^{ab}	0.893^{b}
CE_{30}	8.92^{a}	24.34^{b}	9.673^{ab}	0.961^{a}
CE_{45}	9.01^{a}	28.09^{a}	9.856^{a}	0.980^{a}
CE_{60}	8.07^{b}	21.49^{d}	8.966^{b}	0.887^{b}

Note: Different superscripts in the same column are significantly different between the treatments at 5% level according to Duncan's Multiple Range Test

Table 9. Effect of chitooligosaccharide on the root growth of *Piper nigrum* plantlets after 70 days *ex vitro*

Formulas	Number of root	Length of root (cm)	Fresh weight of root (g)	Dry weight of root (g)
CE_0	12.22^{d}	14.89^{e}	0.129^{c}	0.013^{c}
CE_{15}	14.54^{c}	34.15^{b}	0.399^{b}	0.038^{b}
CE_{30}	19.01^{a}	28.78^{c}	0.521^{a}	0.053^{a}
CE_{45}	12.12^{d}	25.75^{d}	0.474^{ab}	0.049^{a}
CE_{60}	16.89^{b}	34.93^{a}	0.526^{a}	0.053^{a}

Note: Different superscripts in the same column are significantly different between the treatments at 5% level according to Duncan's Multiple Range Test

Fig. 2. Effect of chitooligosaccharide on the root growth of *Piper nigrum* plantlets after 70 days *Ex vitro*

Note: Bar indicates 5 cm

4. CONCLUSION

Based on the obtained results, it can be concluded that sterilize *Piper nigrum* shoots with 30% sodium hypochlorite at 10 minutes was the most suitable condition; appropriate culture media for bud formulation was MS media supplemented 30 g/L saccharose, 7,5 g/L agar, 3 mg/L BA, culture media for growth of plantlet shoot was MS media supplemented 30 g/L sacharose, 7,5 g/L agar, 1 g/L NAA, 2 mg/L IBA and 45 mg/L chitooligosaccharide. Suplementation of chitooligosaccharide at concentration of 45 mg/L was optimal for the growth of *Piper nigrum* plantlets both *in vitro* and *ex vitro.*

ACKNOWLEDGEMENT

This work is supported by a grant of the ministry of Education and Training, Vietnam with titled: “Application of biotechnology for sustainable production of black pepper (*Piper nigrum* L.) in the Central Highland” (code B 2017-2019).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/53376