Effects of Nano Elicitors on Callus Induction and Mucilage Production in Tissue Culture of *Linum usitatissimum* L.

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Abstract

Background: Tissue culture is a new approach for production of secondary metabolites with many advantages over conventional methods. Elicitors such as nano particles are molecules that can stimulate the production of secondary metabolites.

Objective: This study was conducted to evaluate the effects of some nano particles on mucilage production in *Linum usitatissimum* under tissue culture conditions.

Methods: Concentrations of 5, 10 and 20 mg/L of nano-ZnO, nano-SiO_2 and nano-Al_2O_3 were selected as elicitor treatments. The cultures incubated in growth room at 25 ± 1 ºC with 16/8 h illumination periods. After 2 weeks of incubation callus fresh weight, percentage of mucilage and mucilage yield were recorded.

Results: The results indicated the positive effect of low dosage of nano elicitors on callus induction and mucilage production. Furthermore, the callus induction is tightly dependent to the presence of auxin and cytokinin, which stimulate both cell division and cell elongation.

Conclusion: Generally, the type and concentration of nano elicitors had significant effects on the mucilage content.

Keywords: ***Linum usitatissimum*** **L., Callus, Elicitor, Mucilage, Nano particle, Tissue culture**

Introduction

Medicinal plants have a long history of use for therapeutic purposes. *Linum usitatissimum* L., commonly known as Flax is an ancient crop that cultivated for many different uses in industry and herbal medicine. Flaxseed accumulates many biologically active compounds and elements including linolenic acid, linoleic acid, lignans, cyclic peptides, polysaccharides, alkaloids, cyanogenic glycosides, and cadmium. The phytochemical studies revealed that the average composition of commercial seed was 41% fat, 20% protein, 28% total dietary fiber, 7.7% moisture, and 3.4% ash [1]. Flaxseed oil is an excellent source of the omega-3 fatty acid linolenic acid with typical levels of 55% in the oil [2]. Whole flaxseed is widely accepted as a healthy food that has anticancer activity [1]. Flaxseed is an essential source of high quality protein and soluble fiber and has considerable potential as a source of phenolic compounds [2].

Secondary metabolites are valuable phytochemicals, which apparently have medicinal properties and also can be used as taxonomic markers [3]. It is possible to produce secondary metabolites under *in vitro* conditions as an alternative method. Mucilages, which are high molecular weight polysaccharides, are easily extracted from plants and have many applications in folk medicine. Gums and mucilages are classified according to their gummy nature. Mucilages are frequently served in pharmaceutical industries due to their suitable properties such as low cost, availability and non-toxicity. Mucilages are more or less soluble in ware and produce gelatin like substances. Mucilages possess anti-inflammatory, softening and moistening properties [4]. *In vitro* production of secondary metabolites has many advantages over the conventional systems of production from whole plant. This method provides precise control of different factors resulting in unaffected quality of produced compounds over the time. By contrast, the quality of these compounds in plants growing in natural environments strictly influenced by environmental conditions and pests [5].

The use of elicitors is one of the efficient ways to increase the production of secondary metabolites in the plants. Elicitors are biotic or abiotic molecules with stimulating effects on production of plant secondary metabolites in plant, cell and organ cultures [6]. Different types of elicitors have been characterized and used to increase the production of secondary metabolites in plants. Elicitors may be divided into two main groups "biotic and abiotic".

The field of nanotechnology is one of the most active areas of research in modern material science [7]. Nanoparticles are materials that are small enough to fall within the nanometric range with at least one of their dimension beings less than a few hundred nanometers. This reduction in size brings about significant changes in their physical properties with respect to those observed in bulk materials [8]. Nowadays, nanotechnology has many applications in biological science. Water delivery, production of fertilizers and herbicides development, are only some applications of nanotechnology in agriculture [9]. It is generally recognized that silver nanoparticles may attach to the cell wall, thus disturbing cell wall permeability and cellular

respiration. It is also possible to penetrate the nanoparticles within cells and interfere with the phosphorus and sulfur of DNA and proteins [10]. Secondary metabolites present in plant systems may be responsible to nanoparticles as eliciting agents [11]. The previous studies revealed that elicitation by nanoparticles is an effective method in the synthesis of secondary metabolites [12]. Elicitors have also been applied as effective tools to improve the plants protection against pathogen attack [13].

The present study was conducted to evaluate the effects of different nano particles including ZnO, SiO_2 and Al_2O_3 and plant growth regulators on production of mucilage in *Linum usitatissimum* under *in vitro* conditions.

Materials and methods

Explant and Media preparation

The mature seeds of *Linum usitatissimum* L. were sterilized by 70% ethyl alcohol for 1 min. This was followed by surface sterilization in 2.5% sodium hypochlorite solution for 8 min followed by 3 rinses in sterile water. For seed germination, sterile seeds were planted on ½ Murashige and Skoog basal medium [14], supplemented with 30g/L sucrose and 7 g/L agar. The cultures were incubated at growth chamber at 23± 2 ^oC with 16/8 h illumination periods. After seed germination, leaf, stem and root tissues of young plants were taken to use as explants for callus induction. The Culture media were prepared using Murashige and Skoog basal medium salts supplemented with 3% w/v sucrose, 0.7% w/v agar and 1g/L myo-inositol at various concentration of cytokinin and auxins. All growth regulators were added before autoclaving and pH was adjusted to 5.7 ± 0.1. Three types of nano particles including nano-ZnO, nano-SiO_2 and nano-Al_2O_3 were selected as eliciting agents. The nano particles were dissolved in water by ultrasonic homogenizer USH-1200 instrument at room temperature.

Callus induction and elicitor treatment

Five segments of each explants (leaf, stem and root) were cultured into MS medium supplemented with different concentrations of NAA, 2,4-D and BAP (Table 1). The cultures were incubated in growth chamber at 25± 1^oC with 16/8 h illumination periods.

After five weeks, percentage of callus induction, callus diameter (mm) and callus fresh weight (g) were measured. After recording the callus induction percent, callus size and callus fresh weight, high quality calli were selected and sub-cultured on MS medium supplemented with different concentrations of elicitors. Concentrations of 5, 10 and 20mg/L of nano-ZnO, nano-SiO_2 and nano-Al_2O_3 were selected as elicitor treatments. The cultures were incubated in growth room at 25± 1 ^oC with 16/8 h illumination periods. After 2 weeks, callus fresh weight, percentage of mucilage and mucilage yield were recorded. The mucilage content of obtained calli were measured according to the method described by Sharma and Koul [15].

Table 1- Plant growth regulators combinations used in callus induction

Media code	Plant growth regulator combination		
	BAP (mg/L)	2,4-D (mg/L)	NAA (mg/L)
A	1	1	1
B	1	1	-
C	1	-	1
D	1	2	-
E	1	-	2

Statistical analysis

In callus induction experiment, the treatments were arranged in a completely randomized design (CRD) with three replications. For each replication, five explants were placed on the surface of medium. One-way analysis of variance was used for comparison among treatments for callus induction experiment. The data obtained from eliciting experiment were analyzed in a randomized complete block design (RCBD) which explants considered as blocks. Means comparisons were made using Duncan's multiple rang test (DMRT) at 5% level of probability. The statistical analyses were performed with the SPSS statistical software program.

Results

TEM (Transmission electron microscopy) analysis

The size and morphology of the used nano particles were characterized by TEM (Figure 1). The TEM observation indicates all used nano particles have almost spherical structure (Figure 1). The size of nano particles are less than 50 nm.

SEM (Scanning electron microscope) analysis

The Scanning Microscope Electronic (SE) images indicate size and morphology of used nano particles (Figure 2). According these data, all nano particles have a spherical shape with a large surface area.

Effect of plant growth regulators on callus induction

According to the ANOVA (Table 2) the plant growth regulators were significantly different ($P \leq 0.01$) regarding the callus induction, callus size and callus fresh weight. However statistical analysis revealed that these traits were not significantly affected by explants. The produced calli on all tested media were healthy, white in color and compact in texture.

The results revealed that for all traits, the NAA as auxin source was more effective than 2,4-D and the callus induction, callus size and callus weight increased with increasing NAA concentration. Among different combinations of BAP with 2,4-D and NAA, MS media containing NAA were more suitable than 2,4-D supplemented media for callus induction (Figure 3). Maximum callus induction percentage (95%) was recorded at 2 mg/L NAA with a combination of 1mg/L BAP.

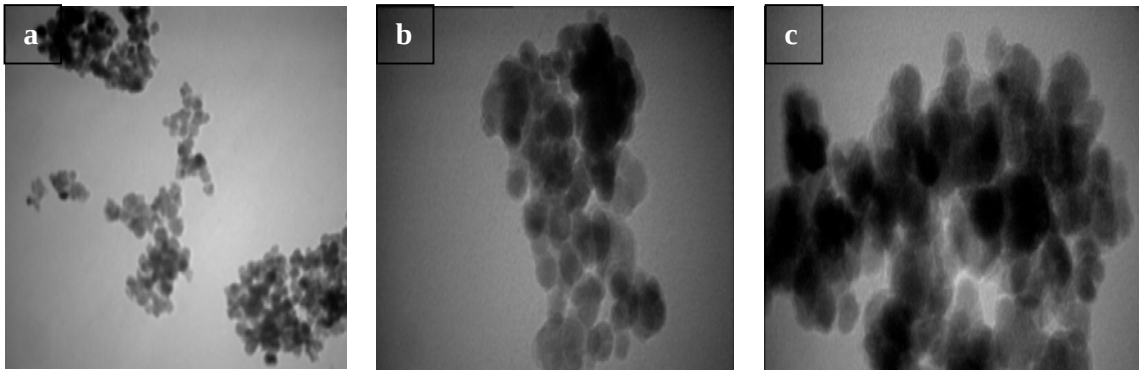


Figure 1- TEM images of (a) SiO_2 (b) ZnO and (c) Al_2O_3.

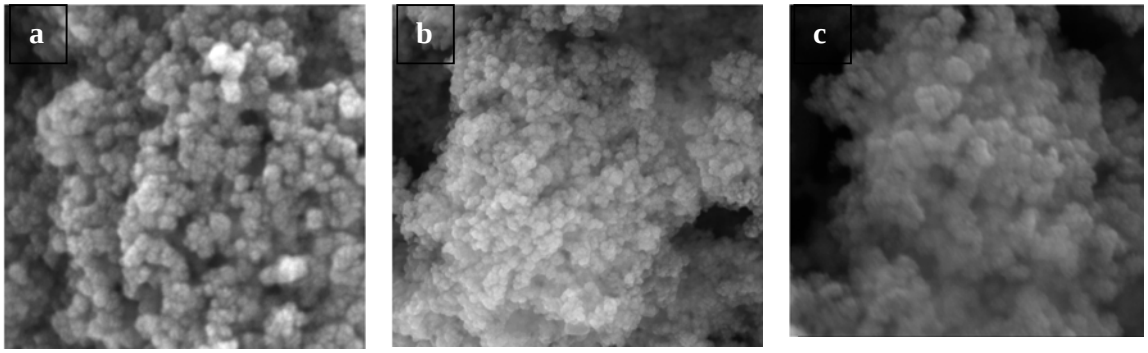
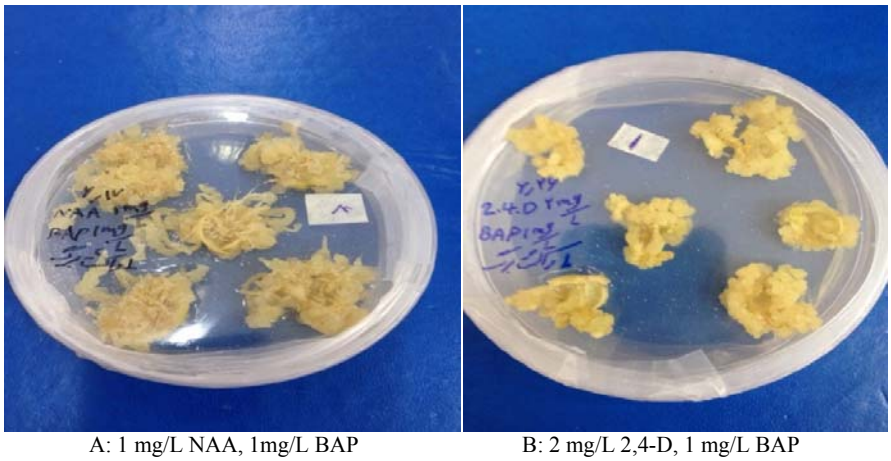


Figure 2- SEM images of (a) SiO_2 (b) ZnO and (c) Al_2O_3.

Table 2- Analysis of variance (ANOVA) for the parameters measured in callus induction.

S.O.V	df	Mean of squares		
		Callus induction (%)	Callus diameter (mm)	Callus fresh weight (g)
Explant	2	0.014^{ns}	0.026^{ns}	0.002^{ns}
Growth regulator	4	0.619^{**}	123.9^{**}	0.257^{**}
Explant × Growth regulator	8	0.03^{*}	0.27^{ns}	0.001^{ns}
Error	30	0.012	0.32	0.002

ns: non significant difference, * and **: significant difference at the 0.05 and 0.01 levels respectively



A: 1 mg/L NAA, 1mg/L BAP B: 2 mg/L 2,4-D, 1 mg/L BAP

Figure 3- Callus production in media supplemented with two different auxins: NAA (A) and 2, 4-D (B).

The highest callus size (17.7mm) was obtained on the MS medium containing 1mg/ L BAP and 2mg/ L NAA and the lowest callus size was recorded for the MS medium supplemented with 2mg/L 2,4-D and 1mg/L BAP. The media containing 2mg/L NAA, 1mg/L BAP) and (2mg/L 2,4-D , 1mg/L BAP) showed the highest and the lowest callus fresh weight respectively (Table 3).

Effect of nano elicitors on callus induction

The results showed that elicitor concentration affected significantly the fresh weight of calluses, but there was no significant difference between types of elicitors for callus fresh weight (Table 4). The average of fresh weight of callus decreased with adding the high amounts of nano elicitors to medium.

Duncan's multiple rang test revealed that supplementation of MS medium with nano-ZnO produced the maximum average (0.62g) of callus fresh weight compared with two other nano particles. The highest callus fresh weight (0.79g) was obtained from MS media supplemented with 5mg/L nano-ZnO (Table 5). The lowest callus fresh weight (0.51, 0.48 and 0.49 for nano-ZnO, nano-SiO_2 and nano-Al_2O_3 respectively) were obtained from the media supplemented with high amounts (20 mg/L) of nano particles (Table 5).

Table 3- Different media effects on calli characteristics of *Linum usitatissimum*.

Media code	Callus induction (%)	Callus diameter (mm)	Callus fresh weight (g)
A	67^{c}	11.5^{d}	0.25^{c}
B	53^{d}	13^{c}	0.22^{c}
C	84^{b}	15.9^{b}	0.39^{b}
D	29^{e}	8.2^{e}	0.13^{d}
E	95^{a}	17.7^{a}	0.56^{a}

Means followed by the same letter in a column are not significantly different at $P < 0.05$ (Duncan test).

Table 4- Analysis of variance (ANOVA) for the parameters measured in eliciting.

S.O.V	df	Mean of squares		
		Callus fresh weight	Percentage of mucilage	Mucilage yield
Explant (Block)	2	0.0001^{ns}	0.149^{ns}	0.032^{ns}
Type of elicitor	2	0.001^{ns}	0.36^{*}	0.187^{*}
Elicitor concentration	2	0.179^{**}	7.9^{**}	6.2^{**}
Type × Concentration	4	0.001^{ns}	0.074^{ns}	0.04^{ns}
Error	16	0.002	0.079	0.038

ns: non-significant difference, * and **: significant difference at the 0.05 and 0.01 levels, respectively

Table 5. Effects of different concentration of elicitors on callus weight and mucilage content.

Type of elicitor	Concentration	Callus fresh weight(g)	Percentage of mucilage (%)	Mucilage yield (mg)
ZnO	5	0.79^{a}	2.8^{a}	22.3^{a}
	10	0.57^{b}	1.8^{cd}	10.3^{c}
	20	0.51^{bc}	0.8^{e}	4.1^{d}
SiO_2	5	0.77^{a}	2.2^{bc}	17.1^{b}
	10	0.58^{b}	1.3^{d}	7.8^{c}
	20	0.48^{c}	0.69^{e}	3.3^{d}
Al_2O_3	5	0.75^{a}	2.6^{ab}	19.8^{ab}
	10	0.58^{b}	1.6^{d}	9.2^{c}
	20	0.49^{c}	0.56^{e}	2.8^{d}

Means followed by the same letter in a column are not significantly different at $P < 0.05$.

The obtained results showed that the type and concentration of nano elicitors had significant effects on the mucilage content. In addition, the interaction of type × concentration of elicitors also had non-significant effect that revealed independent effects of main factors on mucilage production. The impact of different elicitors on mucilage content was different. In the present investigation, the highest (22.3 mg) and the lowest (2.8 mg) levels of mucilage yield were obtained from calli cultured in MS media supplemented with 5mg/L nano-ZnO and 20mg/L nano-Al_2O_3, respectively (Table 5). In addition, the color of calli was changed from yellow to brown with application of elicitors.

Discussion

Based on the results, the use of NAA as the auxin source was more suitable than 2,4-D for callus induction. These results were in agreement with Elgengaihi et al (2006) who reported that the use of NAA as auxin and BAP as cytokinin sources in MS medium, gave the best results for callus induction form *Origanum* species [16].

Increasing in NAA concentration (as auxin source) in combination with BAP (as cytokinin source) in the culture medium had a stimulatory effect on callus induction of all explants. These findings about the positive influence of NAA on callus induction are in agreement with those reported by Karimi et al.(2009) on *Ducrosia flabellifolia* [17]; Irvani et al. (2010) on *Doremam moniacum* [18]; Barakat et al. (2011) on *Gypsophila paniculata* [19] and Bernard et al. (2007) on *Ferula gummosa* [20]. These result revealed that callus induction is tightly dependent to the presence of auxin and cytokinin, which stimulate both cell division and cell elongation [21].

Some metal ions, such as zinc, have been reported to influence secondary metabolites production [22]. Elicitation in a proper concentration and at a suitable time can shorten the time needed to achieve the highest level of metabolites [23]. Our data indicated a negative correlation between nano particle concentration and callus fresh weight. The metal ions play an

important role in the activities of proteins involved in maintaining the growth of organisms, but, at high concentrations they are harmful to living organisms [24]. The callus growth was inhibited by adding high levels of nanoelicitors as reported by Tassoni et al. (2005) [25].

The reduction of callus production after adding high amounts (20 mg/L) of nano particles might be attributed to the stress in the medium by these agents on cell growth and cells division. Both positive and negative effects of nano particles on plant growth have been reported. The results of some studies have shown inhibitory effects of nano-oxide materials such as Cu, Al, Si, Fe and Zn on development of plant growth [26, 27, 28]. Metal ions such as nickel, cobalt, zinc, and manganese are necessary for regulating enzyme activity despite being highly toxic at high concentrations [29].

Considering the ability of callus tissue to produce mucilage, tissue culture methods can be used as a suitable way for mucilage production under controlled conditions. The mucilage is regarded as normal physiological product of metabolism formed within the cell or deposited on it in layers. Mucilages in plants are thought to aid in water storage and seed germination, and to act as a membrane thickener and food reserve. Natural gums and mucilages have been widely explored as pharmaceutical excipients [30]. The result of present study was in agreement with the results of Fakruddin et al [31] which reported that nanoparticles have a good potential to be used as effective elicitors in plant biotechnology.

Conclusion

Generally, the results of present study revealed a positive effect of low dosage of nano zinc oxide as a nano-elicitor on callus induction and mucilage production in *Linum usitatissimum*. The application of nano particles to elicit the production of secondary metabolites is still in its early stages and more complementary studies are needed to evaluate the effectiveness, risks and safety factors of nano elicitors.

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