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# Enhancement of Callus Growth and Hyoscyamine Alkaloid Production in *Hyoscyamus muticus* by Nanotechnology, Biotic Elicitor and Precursor

**Hussein Bosila, Mohamed Abdelfattah Hamza* and Ahmed Abd El-Fattah El-Ateeq**

**Department of Horticulture, Faculty of Agriculture, Al Azhar University, Cairo, Egypt**
***Corres. author: hamza.plant@gmail.com**

**Abstract :** This study aimed to determinate the optimal condition for mass production and hyoscyamine alkaloid content in *in vitro* callus cultures of *Hyoscyamus muticus* under different treatments of nanomaterial compound (Lithovit 0.25, 0.5, 0.75 and 1 g/l), biotic elicitor (Yeast extract 0.25, 0.5, 0.75 and 1 g/l ) and precursor (Phenylalanine 10, 50, 100 and 200 mg/l). Data show that efficient use of the tested substances to stimulate the callus growth revealed , five levels of different examined substance to achieve the highest significant results due to yeast extract (0.25g/l–7.09 g/explant) and (0.75g/l -7.95 g/explant), (lithovit, 0.25g/l - 6.95 g/explant) and phenylalanine (10 mg/l - 6.95 g/ explant) and (50 mg/l - 7.16 g/ explant) in callus fresh weight. Three of significant results in callus dry weight agreed with callus fresh weight as follows, yeast extract (0.75 mg/l. – 0.18 g/ explant), lithovit (0.25 g/l – 0.2 g/ explant) and phenylalanine (50 mg/l- 0.18 g/ explant), while yeast extract (1.0 g/l.) achieved highest significant value (0.18 g/explant) without agreement with callus fresh weight. The highest value of hyoscyamine alkaloid content (3.01 mg/g dry weight) was recorded with phenylalanine at 200 mg /l.
**Key words:** *Hyoscyamus muticus*, lithovit, phenylalanine, yeast extract and callus.

## Introduction

Medicinal plants are the local heritage with global importance and world is endowed with a rich wealth of medicinal plants [1]. Plant considers one of the most important sources of medicines for thousands of years. Even today, the World Health Organization estimates that up to 80% of people still rely mainly on traditional remedies such as herbs which contain different active ingredients. Biotechnological tools are important for multiplication and genetic manipulation of the medicinal plants through callus inductions, cell suspension in bioreactors, *in vitro* regeneration of plantlets and genetic transformations [2]. *Hyoscyamus muticus* L. (Egyptian henbane) is a member of the family *Solanaceae*, which is one of the large drug producing families [3]. It is famous for its tropane alkaloid content, the main alkaloids present are scopolamine and hyoscyamine. The effects of these alkaloids include stimulation of the central nervous system and simultaneous depression of the peripheral nerves typical for a parasympathomimetic. The medicinal uses include spasmolytic, antiasthmatic, anticholinergic, narcotic and anaesthetic properties [4].

Nanotechnology is an exquisite field in modern material sciences and it is currently an intense area of interest for research [5]. A nanometer is one-billionth of a meter, too small to be seen with a conventional lab

microscope. It is at this size scale, about 100 nanometers or less, that biological molecules and structures inside living cells operate [6]. Lithovit, natural $CO_2$ foliar fertilizer, is a new nanotechnological fine powder created by tribodynamic activation and micronization. In addition, the micronutrients also contained in the product and the trace elements that influence plant physiology, such as manganese, copper, zinc etc. increase the resistance, growth, vitality and quality of the crop. Lithovit is 100 % organic calcite carbonate from natural limestone deposits, suitable for use in organic farming in the European Community, harm-less to humans and animals and not hazardous to water [7]. Nanomaterials because of their tiny size show unique characteristics. They can change physic–chemical properties compared to their bulk materials, they have a great surface area than bulk materials. Because of these larger surface areas, their solubility and surface reactivity was higher [8, 9].

Elicitations are considered to be an important strategy towards improved *in vitro* production of secondary metabolites. Various biotic and abiotic factors added to the medium of callus production influence their production by activating genes for de novo synthesis or by stimulation the physiological processes leading to enhanced accumulation of such products [10]. Amino acids play an important role in alkaloid metabolism. It was demonstrated that they exert multifaceted effects on alkaloid accumulation [11]. The effects of exogenous amino acids could be determined by their increased endogenous levels due to the control of activities of corresponding enzymes of secondary metabolism or due to the control of the biosynthesis of these enzymes themselves [12].

The aim of this work was to study the effect of nanomaterial compound (Lithovit), elicitor (Yeast extract) and precursor (Phenylalanine) on callus growth and hyoscyamine alkaloid content of *H. muticus* L.

## Materials and Methods

This study was conducted in Biotechnology Lab., Horticulture Department, Faculty of Agriculture, Al-Azhar University, Cairo.

**Establishment of callus culture:**

Seeds of *H. muticus* were washed several times with commercial detergent and tap water, and surface sterilized with 70% ethanol for 30 sec., followed by 20% commercial Clorox (contained 5.25% sodium hypochlorite) with a few drops of Tween 20 for 20 min., then rinsed three times in sterile distilled water to remove the residual sodium hypochlorite. After sterilization, seeds were sowed in solid MS basal medium [13] with 30 g/l sucrose for *in vitro* germination. For callus induction, leaf segments (0.5 $cm^2$) were excised from *in vitro* seedling (Fig. (1)A) and placed on solid MS medium supplemented with 0.5 mg/l each of BA and NAA, and 30 g/l sucrose as reported with Aly *et al.* [14]. The pH value was adjusted to 5.8 before added 2 g/l phytagel and autoclaved at 121 °C for 20 min.

**Effect of nanomaterial, elicitor and precursor treatments**:

To test the effect of nanomaterial compound (lithovit), elicitor (yeast extract) and precursor (phenylalanine) on callus growth and alkaloid production in *H. muticus,* callus pieces (0.8 g) taken from leaf callus in the age of four weeks (Fig. (1)B) were transferred to MS medium containing 30 g/l sucrose, 0.5 mg/l each of BA and NAA, and supplemented as follows:

1. Yeast extract (YE) at 0.25, 0.5, 0.75 and 1 g/l
2. Lithovit ( LV) at 0.25, 0.5, 0.75 and 1 g/l
3. Phenylalanine (PHE) at 10, 50, 100 and 200 mg/l

**Culture conditions:**

The different treatments were incubated for four weeks in growth chamber under 25±2$^oC$ and 16-h light/8-h dark cycle with illumination from cool white fluorescence lights 40 $\mu mol^{-2}\ s^{-1}$.

**Measurements and determinations:**

After four weeks incubation period the following data were recorded:

**1. Callus fresh weight (g/explant)**, was determined by weighing callus immediately after remove the residual phytagel.

**2. Callus dry weight (g/explant)**, was determined after drying in an oven at 65$^{o}$C until constant weight.

**3. Total alkaloids (Hyoscyamine Alkaloid mg/g dry weight)** content, was determined spectrophotometry according to the methods of [15,16]. This method is based on the reaction between alkaloid and bromocresol green (BCG).

**The statistical analysis:**

Data of each trial were analyzed separately using one way analyses of variances according to SPSS program [17] where appropriate treatment means were separated using the Duncan´s Multiple Range Test.

## Result and discussion

### Callus fresh weight

Data in Table (1) and Fig. (2) show that yeast extract (as elicitors) achieved positive effect on callus fresh weight when compared with the rest treatments (lithovit and phenylalanine), while phenylalanine recorded the lowest significant values. The results indicate that control treatment recorded moderate value (6.37 g/explant). Yeast extract, 0.25g/l and 0.75 g/ l achieved significant effect on callus fresh weight (7.09 and 7.95 g/explant, respectively). Increasing levels of lithovit (0.25, 0.5, 0.75 and 1.0 g/l) achieved gradually decreasing in callus fresh weight (6.95, 5.41, 4.47 and 4.59 g/explant, respectively). Respecting phenylalanine study, the first and second level (10 and 50 mg/l) recorded significant values (6.96 and 7.16 g/explant), while at higher levels (100 and 200 mg/l) recorded very weak values in callus fresh weight (5.47 and 3.88 g/explant, respectively).

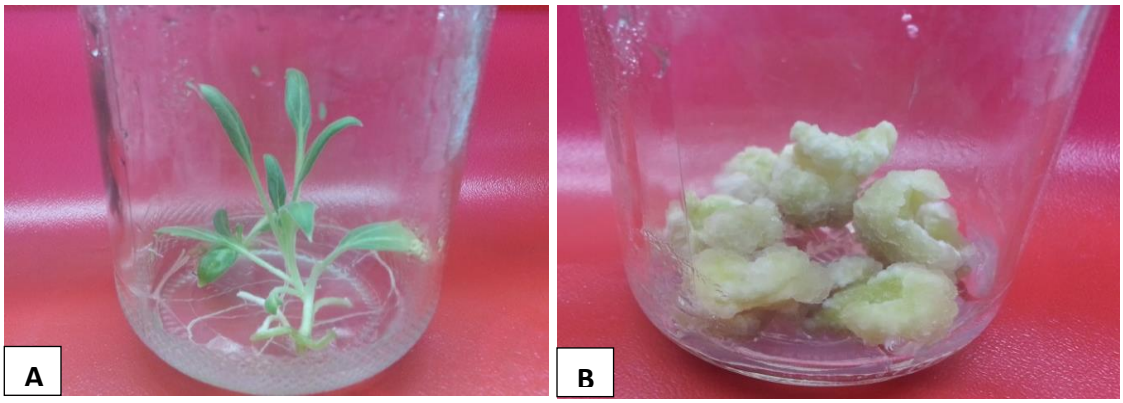


**Fig. (1) A. *In vitro* seedling of *H. muticus*, B. Leaf callus established on MS medium supplemented with 0.5 NAA + 0.5 BA mg/l after four weeks incubation periods.**

**Table (1) Effect of yeast extract, lithovit and phenylalanine on callus production and alkaloid content (hyoscyamine) in *H. muticus*.**

| Treatments | | Callus fresh weight g/explant | Callus dry weight g/explant | Alkaloid content mg/g dry weight |
|---|---|---|---|---|
| Control | 0 | 6.37 bc | 0.14 ef | 1.60 |
| Yeast extract (YE) g/l | 0.25 | 7.09 ab | 0.15 de | 1.73 |
| | 0.5 | 6.36 bc | 0.15 ef | 1.20 |
| | 0.75 | 7.95 a | 0.19 ab | 1.57 |
| | 1 | 6.13 bc | 0.18 abc | 1.17 |
| Lithovit (LV) g/l | 0.25 | 6.95 ab | 0.20 a | 1.49 |
| | 0.5 | 5.41 cd | 0.16 cde | 2.00 |
| | 0.75 | 4.47 de | 0.15 ef | 1.57 |
| | 1 | 4.59 de | 0.14 ef | 1.22 |
| Phenylalanine (PHE) mg/l | 10 | 6.92 ab | 0.17 bcd | 1.49 |
| | 50 | 7.16 ab | 0.18 abc | 2.29 |
| | 100 | 5.47 cd | 0.14 ef | 2.61 |
| | 200 | 3.88 e | 0.13 f | 3.01 |

Mean values in each column with the same letter are not significantly different at $p<0.05$ by Duncan's multiple range test.

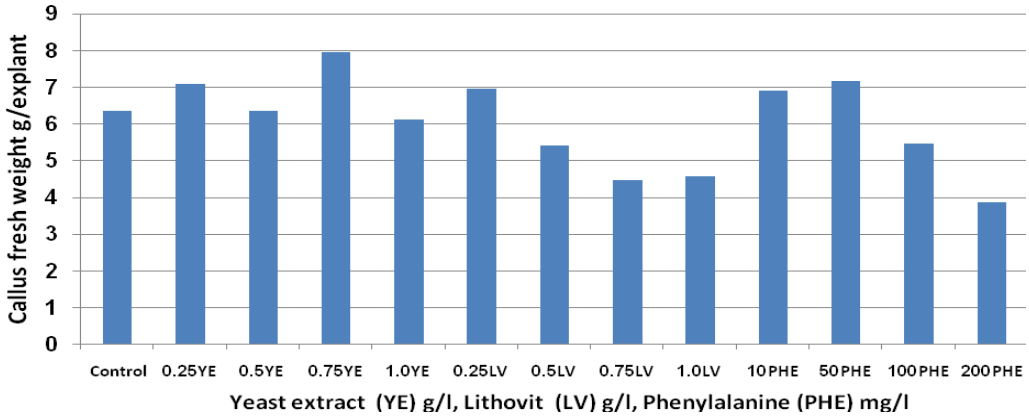


**Fig. (2) Effect of yeast extract, lithovit and phenylalanine levels on callus fresh weight of *H. muticus* after four weeks incubation periods.**

**Callus dry weight**

Data in Fig. (3) and Table (1) revealed that control treatment recorded very weak value (0.14 g/explant) of callus dry weight. Yeast extract at low and moderate levels (0.25 and 0.5 g/l) caused the same value of callus dry weight (0.15 g/explant), also (0.75 and 1.0 g/l levels) formed the same value with increasing of callus dry weight (0.18 g/explant). Callus dry weight negatively correlated with increasing lithovit concentration, as 0.25, 0.5, 0.75 and 1.0 lithovit g/l recorded 0.20, 0.16, 0.15 and 0.14 g/explant, respectively. In this concern, 0.25 g/l achieving the highest significant value absolutely. Phenylalanine levels (10 and 50 mg/l) caused slightly

significant value on callus dry weight, while higher levels (100 and 200 mg/l) achieved significant decreasing in callus dry weight (0.14 and 0.13 g/explant, respectively).

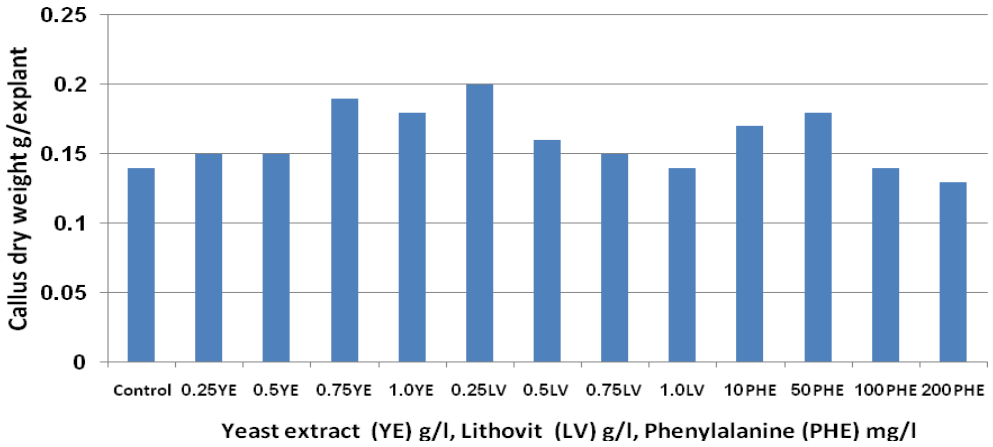


**Fig. (3) Effect of yeast extract, lithovit and phenylalanine on callus dry weight of *H. muticus* after four weeks incubation periods.**

**Alkaloid content**

From the presented data in Fig. (4) and Table (1) it appears that the tested material achieved positive effect on hyoscyamine alkaloid content when compared to the control treatment which recorded moderate value (1.60 mg/g dry weight). The highest value of hyoscyamine alkaloid content (3.01 mg/g dry weight) was formed in callus with phenylalanine at 200 mg /l. Yeast extract at different levels (0.25, 0.5, 0.75 and 1.0 g/l) gave fluctuating results as follows 1.73, 1.20, 1.57 and 1.17 mg/g dry weigh, respectively. With respect to lithovit, hyoscyamine levels increased (1.49 and 2.00 mg/g dry weight) with increasing lithovit levels from 0.25 to 0.5 g/l, respectively), and decreased at 0.75 and 1.0 g/l (1.57 and 1.22 mg/g dry weight, respectively). Increased phenylalanine levels (10, 50, 100 and 200 mg/l) resulted in a super increase in hyoscyamine alkaloid content as follows; 1.49, 2.29, 2.61 and 3.01 mg/g dry weight, respectively.

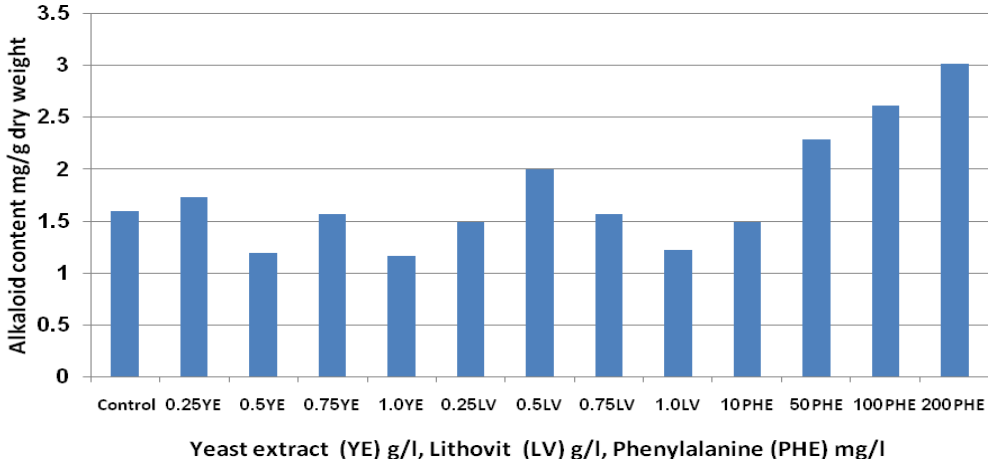


**Fig. (4) Effect of yeast extract, lithovit and phenylalanine on alkaloid content (hyoscyamine) of *H. muticus* after four weeks incubation periods.**

Callus is a mass of unorganized parenchyma cells derived from plant tissues and induced from plant tissues after plating onto *in vitro* special tissue culture medium [18]. Callus culture of medicinal plants is one of the ways for production of secondary metabolites [19-23].

Our results interpret as follow; phenylalanine gave positive results in all data recorded by [24] in *Taxus chinensis* and [25] in *Taxus baccata*; in *Taxus baccata*, addition of phenylalanine increased the Taxol amount higher than optimum concentration for flavonoid production. Feeding experiment (phenylalanine) on the production of isoflavones in *Psoralea corylifolia* hairy root culture demonstrated that phenylalanine increased the production of daidzein and genistein by 1.3 fold compared with the control [26]. Phenylalanine successfully triggered the production of flavonoid by 23% higher than the control [27]. Flavonoid originated from phenylalanine, an upstream metabolic precursor through phenylpropanoid pathway. Considering this phenylalanine supplementation which is expected to increase the metabolic flux through phenyl-propanoid biosynthetic pathway and elevate the level of targeted compound. Phenylalanine supplementation has been reported to enhance secondary metabolite production in plant cell cultures [26].

In lithovit treatments, given the composition of lithovit ($CaCo_3$ 79.2 %) as nanotechnology component. Examples materials of nanomaterials carbon what follows : (single-walled carbon nanotubes (SWCNTs) , multi-walled carbon nanotube (MWCNTs) and etc.), the attachment of (MWCNTs) to the proteins and polysaccharides of cell wall results in a signaling cascade which increased the production of certain compounds necessary for cell wall thickening, also plants provide a potential pathway for the transport of nanoparticles to the environment and serve as an important route for their bioaccumulation into food chain [28]. Plant cell wall acts as a barrier for easy entry of any external agent including nanoparticles into plant cells. The sieving properties are determined by pore diameter of cell wall ranging from 5 to20 nm [29]. Hence, only nanoparticles or nanoparticle aggregates with diameter less than the pore diameter of the cell wall could easily pass through and reach the plasma membrane [30]. There is also a chance for enlargement of pores or induction of new cell wall pores upon interaction with engineered nanoparticles which in turn enhance nanoparticle uptake. Further internalization occurs during endocytosis with the help of a cavity like structure that form around the nanoparticles by plasma membrane. They may also cross the membrane using embedded transport carrier proteins or through ion channels. In the cytoplasm, the nanoparticles may bind with different cytoplasmic organelles and interfere with the metabolic processes at that site [31]. Accumulation of nanoparticles on photosynthetic surface cause foliar heating which results in alterations to gas exchange due to stomatal obstruction that produce changes in various physiological and cellular functions of plants [32]. The mechanism of formation of nanoparticles; whether they are formed outside in the media and then translocated to plants or whether they are formed by the reduction of metal salts within the plants itself still needs more clarification [33] The uptake and translocation of nanoparticles across root cells [in which several active and passive transport processes involve] depends on the type of metal ions and plant species. The amount of nanoparticle accumulation in plants also varies with reduction potential of ions and the reducing capacity of plants that depends on the presence of various polyphenols and other heterocyclic compounds present in plants [34]. Tantawy *et al.* [9] found that nano silicon is more effective and efficient compared to regular silicon application in mitigating salt stress damages on sweet pepper plants.

Concerning yeast extract, addition yeast extracts were used as growth nutrients such as crown-gall tissue cultures and callus cultures [35, 36]. George *et al.* [37]suggested that yeast extract is used as a supplement in order to promote plant growth, due to its high amino acid content. However, different species respond in different ways to the presence of yeast extract that is, addition of higher concentration of yeast extract to MS medium, inhibit the growth whereas, lower concentration of yeast extract was found beneficial [36]. On *Curcuma mangga*, Abraham *et al.* [38] found that yeast extract used as supplement in proliferation medium did not affect the shoot proliferation and inhibition of leaf-development of *in vitro* plantlets and also inhibition of leaf-development and did not affect the fresh and dried biomass of the plantlets also yeast extract in the *in vitro* plantlets.

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