Silver nitrate regulating phenolic acid and flavonoid accumulations in callus cultures of *Aerva lanata*, an important medicinal herb

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Abstract

In vitro cultures of the medicinal herb, *Aerva lanata* exhibited heterogeneity in diverse concentrations of (0.5-10 mg/l) silver nitrate. The texture of the callus tissue varied from friable, non-friable to nodular. Callus cultures also exhibited a color variation ranging from white, brown to black. Silver nitrate did not exhibit a significant variation in the accumulation of in vitro biomass at a concentration range of 0-5 mg/l. Higher concentrations were found inhibitory for callus growth. The cultures exhibited a positive correlation between silver nitrate concentration and accumulation of phenolics as well as flavonoids up to a concentration of 5.0 mg/l.

The results generated clearly indicated that silver nitrate may be used as an additive for regulating the callus morphology, growth and accumulation of phenolic compounds under in vitro conditions.

Keywords: *Aerva lanata*, Callus culture, Flavonoids, Phenolic compounds, Silver nitrate.

Introduction

Silver nitrate is an inorganic compound which is well known for its astrangent and antiseptic properties. Chemically it exists as a colorless white crystal which is sensitive to sunlight. When exposed to sunlight, it changes its color to brown because the light induced chemical reaction. In animal cells, the silver ion is released and precipitated as chlorides of phosphates or inert protein complexes and is responsible for acting as a toxic factor. In plants, silver nitrate is used as a regulating factor for in vitro morphogenesis for direct and indirect regeneration. There are few reports available regarding the in vitro synthesis of secondary metabolites such as vanillin in *A. lanata*. Therefore in vitro culture of *A. lanata* is an ideal system to study the effect of silver nitrate on the synthesis of phenolics and flavonoids.

Material and Methods

Preparation of media, sterilization and maintenance of cultures: The stock plants of *A. lanata* were maintained under the greenhouse of Department of Biotechnology, Manipal Institute of Technology and served as the stock plants. Young green leaf segments from this plant were washed with running tap water for 30 minutes and then sterilized with 0.1% mercuric chloride for 8 minutes. Further, the explants were washed twice with autoclaved double-distilled water and the cut ends were removed. The explants after post sterilization were inoculated into Woody Plant Medium (WPM) containing 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) 1 mg/l kinetin and 1 mg/l indole acetic acid (IAA).

For experiments, silver nitrate was added at a concentration range of 0.5 to 10 mg/l. The media pH was adjusted to 5.80 and steam sterilized in an autoclave at a temperature of 121°C for 15 minutes. Subculturing was done after 21 days. The cultures were incubated under 12 h photoperiod at a temperature of 28+2°C. All the experiments were carried out in three replications. The cultures devoid of silver nitrate served as the control.

Quantitative estimation of total phenols: The analysis of the total phenols and flavonoids was done after 42 days of callus growth. The quantitative estimation of phenolic compounds was analyzed by following the procedure of Folinis method. The callus tissue was dried in an oven at 30°C for 24 hours to record the dry weight of the sample. The dried callus tissue was extracted with ethanol using a Soxhlet apparatus. The extract was centrifuged at 12000 g for 20 minutes, and the supernatant was used for further analysis of free phenolics. The supernatant was evaporated to dryness, and the residue was dissolved in double distilled water. 0.5 ml of Folin-Ciocalteau reagent was mixed well to serve as the control.
room temperature and the absorbance was recorded at 650 nm measured against a blank. Standard graph of absorption was prepared by using different concentrations of chlorogenic acid. The results of the total phenol estimation were calculated as chlorogenic acid equivalents.

**Quantitative analysis of flavonoids:** For the quantification of flavonoids, the method of well-established aluminium chloride colorimetric method was followed. For this, the sample extracts were allowed to react with 0.6 ml of 2% aluminium chloride. After incubating for one hour at room temperature, the absorbance was recorded against a blank at 420 nm. Standard solution of quercetin served as the standard solution for preparing the standard curve.

**Results and Discussion**

A concentration of 5 mg/l silver nitrate induced maximum accumulation of phenolic compounds and flavonoids. However, callus growth was maximum when 0.5 mg/l silver nitrate was added into the medium (Table 1). This indicated that the presence of silver nitrate is not beneficial in inducing cell division and growth in callus cultures of *A. lanata*. On the other hand, silver nitrate is inducing a stress response as evidenced by reduced callus growth and increased total phenolics as well as flavonoids accumulation with increasing silver nitrate concentration.

In a different experiment in wheat, it was revealed that silver nitrate is beneficial in increasing callus growth and embryo differentiation. The response of the wheat also exhibited a strong genotypic effect. Similar to the present study, a low concentration of silver nitrate 0.2 mg/l was beneficial in callus formation in *Solanum nigrum* explants. This study also revealed that the nature and color of the callus tissue vary with different concentration of silver nitrate. In another study involving *Zinnia* cultivars, it was found that the presence of silver nitrate reduces callus growth. The existing reports indicate a positive correlation of silver nitrate and callus formation while few other reports indicated that silver nitrate is not favorable for callus induction and growth. The present study could not demonstrate a strong influence of silver nitrate in callus induction and growth of explants of *A. lanata*. Hence it may be concluded that the effects of silver nitrate are genotype-dependent, and it may not be possible to compute precisely as the results varied from plant to plant.

A concentration of 2.0 mg/l silver nitrate was suitable for phenolic acid accumulation in callus cultures of *Paeonia ostii*. The results of the current experiments revealed a positive correlation between silver nitrate concentration and phenolic accumulation in callus cultures. However, the results generated here are contrary to the results obtained with cell suspension cultures of *Capsicum annuum* where silver nitrate reduces phenolic compound accumulation. The use of silver nitrate in regulating phenolic leaching and avoiding the cell death in callus was demonstrated in the cultures of *Passiflora edulis*. According to Park et al., treatment of high concentration of silver nitrate (30 mg/l) in the presence of 500 mg/l yeast extract elicited phenylpropenoid pathway and rosmarinic acid synthesis in *in vitro* cell cultures of *Agastache rugosa*. Similar results were also observed in hairy root cultures of *Salvia miltiorrhiza*. Osman et al. experimented the use of silver nitrate in the elicitation of gallic acid in *Barringtonia racemosa* cell suspension cultures.

Varying concentrations of silver nitrate also induced diverse callus morphology. Low concentrations of silver nitrate at a range of 0.5 to 1.0 mg/l was suitable for inducing whitish powdery, friable callus without any dark pigmentation (Fig. 1a, b). Higher concentrations of silver nitrate (2-10 mg/l) induced dark brown to black color (Fig. 1c-g). In many cultures, some regions of the dark callus were showing white powdery active growing regions (Fig. 1c-e). Silver nitrate also induced some changes in the callus induction.

### Table 1

**Callus growth and the yield of total phenolics and total flavonoids with respect to diverse concentrations of silver nitrate added to WPM containing 2mg/l 2,4-D, 1 mg/l each of Kinetin and IAA.**

**Values are the mean of three replications.**

<table>
<thead>
<tr>
<th>Silver nitrate (mg/l)</th>
<th>Callus Dry wt (mg)</th>
<th>Total phenolics (µg catechol equivalent/g of dry sample)</th>
<th>Total Flavonoids (µg quercetin equivalent/g of dry sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>120</td>
<td>3.04</td>
<td>8.573</td>
</tr>
<tr>
<td>0.5</td>
<td>135</td>
<td>8.13</td>
<td>12.62</td>
</tr>
<tr>
<td>1.0</td>
<td>129</td>
<td>11.85</td>
<td>14.32</td>
</tr>
<tr>
<td>1.5</td>
<td>126</td>
<td>14.92</td>
<td>17.14</td>
</tr>
<tr>
<td>2.0</td>
<td>131</td>
<td>20.73</td>
<td>18.49</td>
</tr>
<tr>
<td>2.5</td>
<td>128</td>
<td>41.23</td>
<td>20.22</td>
</tr>
<tr>
<td>3.0</td>
<td>119</td>
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<td>22.27</td>
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<tr>
<td>5.0</td>
<td>108</td>
<td>98.64</td>
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</tr>
<tr>
<td>7.0</td>
<td>83</td>
<td>65.23</td>
<td>20.58</td>
</tr>
<tr>
<td>10</td>
<td>71</td>
<td>52.58</td>
<td>18.98</td>
</tr>
</tbody>
</table>
Higher concentrations resulted in callus formation restricted towards the cut ends (Fig. 1d-f). Very high concentration (10 mg/l) was inhibitory for callus induction and subsequent growth (Table 1, Figure 1g). Callus cultures of Solanum nigrum also exhibited color variations in the presence of silver nitrate ranging from green to brown. Morphologically the callus cultures also exhibited fragile to nodular. The current study also exhibited nodular callus when the cultures were incubated at a higher concentration of silver nitrate (Fig. 1e). Nodular callus was also observed in cultures of Buchloe dactyloides in the presence of silver nitrate. The results of the current experiment point to the fact that silver nitrate can be used to induce desired morphometric or colour variations in the callus.

The results of the experiments conducted by Vain et al. and Songstad et al. also confirmed the morphological changes of callus formed from monocots with respect to silver nitrate.

**Conclusion**

The current experiments in callus culture of A. lanata revealed that silver nitrates possessed an influence in the callus morphology, growth as well as accumulation of plant phenolics and flavonoids. Therefore, silver nitrate can be considered as an effective regulator for controlling in vitro growth and metabolism of plant cells and tissues. The results generated here are further interesting towards identifying the exact mechanism of action of silver nitrate in plant cells and its metabolism.

**References**


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