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Effect of silver nitrate on shoot multiplication, rooting induction and plantlet characteristics of St. John's wort (*Hypericum perforatum* L.) *in vitro* culture

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St. John's wort (*Hypericum perforatum* L.) is one of the introduced medicinal plants that have medicinal uses for anti-depressant. Germplasm conservation of St. John's wort has been conducted at the laboratory for over ten years and plantlets showed a rosette growth. Therefore, laboratory investigations are necessary to be done to have better methods for obtaining normal growth of culture. In this study, we evaluate the effect of concentration levels of silver nitrate ($AgNO_3$) on shoot multiplication, rooting induction and visual characteristics of plant *in-vitro* culture. The study was conducted in two stages (shoot multiplication and rooting induction). For shoots multiplication, cultures were grown in Murashige and Skoog (MS) media supplemented with 0.1 mgl^{-1} N^6-Benzyl Adenine (BA) combined with various concentration levels of $AgNO_3$ as follow: MS + 0.1 mgl^{-1} BA + $AgNO_3$ (0.0, 0.1, 0,3, 0.5 and 0.7 mgl^{-1}). For rooting induction, cultures were grown in half-formula of MS media with various concentration levels of $AgNO_3$: ½ MS + $AgN0_3$ (0.1, 0.3, 0.5 and 1.0 mgl^{-1}). The results showed that the application of $AgNO_3$ combined with 0.3 mg/l BA could improve the culture characteristics and showed normal plantlets. The best rooting induction was obtained at ½ MS + 0.3 mgl^{-1} $AgNO_3$. This protocol provides a technique for improving visual culture during conservation.

Key words: *Hypericum perforatum* L., shoot multiplication, protocol for improving visual culture, *in vitro*.

INTRODUCTION

St. John's wort (*Hypericum perforatum* L.) is one of the medicinal plants that have been used for over a decade (Gadzovska et al., 2012). St. John's wort is a species of the *Hypericaceae* that has many benefits and efficacy as medicinal treatments such as burns, bruises, swelling, wound healing, mild to moderate anti-depressant, antiviral, antibiotic, antioxidant and anti-cancer (Luo et al., 2004; Agostinis et al., 2002; Silva et al., 2005; Yadollah-Damarvandi et al., 2015).

St. John's wort grows optimally in the highlands. Traditionally, plant propagation can be done by separating tillers, or generatively by seeds. St.John's wort needs to be conserved because of the benefit of this plant as medicine and plant germplasm need to be maintained for future research purpose. To support the conservation of St. John's wort germplasm in the

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Indonesian Spice and Medicinal Crops Research Institute (ISMCRI-IAARD), propagation is done through tissue culture technique. Plantlets multiplication is done by using Murashige and Skoog (MS) media, supplemented with 0.1 mgl^{-1} BAP. In ISMCRI-IAARD, St. John's wort has been conserved as *in vitro* culture for over ten years. The culture grew on MS media supplemented with 0.1 mgl^{-1} BA, and has fairly characteristics of their acclimated plants. It showed rosette growth, small plant leaves, no roots, and the ones which are not the original characteristics of normal plants as grown in the field. Those abnormal characteristics may be due to changes during culture for a long period.

Many factors may influence plant characteristics during its growth in the *in vitro* culture, which tends to produce changes at morphological, cytologycal, physiological, biochemical or event at molecular levels (Bajaj, 1992). Changes that occur during the period of *in vitro* culture can be triggered by both internal and external factors, such as; sources of explants, media composition, type and concentration of growth regulators used (Karp, 1991; Veilleux and Johnson, 1998). In case of propagation of Big-White Ginger Cultivar of Indonesian Variety Cimanggu-1 *in vitro* by direct organogenesis, the healthy plantlet and vigorous plants were not able to produce normal rhizome after planting in the field. It is suggested that genetic alteration or epigenetic change during the *in vitro* culture and regeneration have been performed (Rostiana and Syahid, 2008).

Silver nitrate ($AgNO_3$) is an inhibitor of ethylene activity and has been widely used in *in vitro* plant propagation. This chemical compound also plays a role in the process of shoot proliferation and multiplication, as well as rooting induction in *Solanum nigrum* culture (Geetha et al., 2016), and induced shoot multiplication and rooting of vanilla (*Vanilla planifolia*) (Giridhar et al., 2001). The application of silver nitrate showed the effect to increase direct organogenesis from leaf explants of *Brassica napus* and *Sinningia speciosa* (Akasaka et al., 2005; Park et al., 2012).

The purpose of this study was to determine the effect of concentration levels of silver nitrate on shoot multiplication, root induction and visual characteristics of St. John's wort *in-vitro* culture.

MATERIALS AND METHODS

Plant materials and culture conditions

St. John's wort has been conserved as *in vitro* culture for over ten years. The *in-vitro* culture was prepared from a sterile young shoot taken from two months old of healthy St. John's wort plant. It is transferred and grown in MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose.

Shoot multiplication culture was performed on MS media supplemented with N^6-benzyl adenine (BA), in combination with concentration levels of silver nitrate ($AgNO_3$): 1) MS + 0.1 mgl^{-1} BA + 0.0 mgl^{-1} $AgNO_3$0, 2) MS + 0.1 mgl^{-1} BA + 0.1 mgl^{-1} $AgNO_3$ 0.1, 3) MS + 0.1 mgl^{-1} BA + 0.3 mgl^{-1} $AgNO_3$, 4) MS + 0.1 mgl^{-1} BA + 0.5 mgl^{-1} $AgNO_3$, and 5) MS + 0.1 mgl^{-1} BA + 0.7 mgl^{-1} $AgNO_3$. Plantlets produced a highest number of shoot multiplication then were used for rooting induction experiment. Rooting induction culture was prepared on a half-strength of MS media, in combination with concentration levels of $AgNO_3$ as follow: 1) ½ MS + 0.1 mgl^{-1} $AgN0_3$, 2) 1/2 MS + 0.3 mgl^{-1} $AgNO_3$, 3) 1/2 MS + 0.5 mgl^{-1} $AgNO_3$, and 4) 1/ 2MS + 0.5 mgl^{-1} $AgNO_3$. Number of shoots produced, shoot length, number of leaves, root number and length, and plantlets characteristics were observed during treatment. Growing plantlets of St. John's wort were maintained in *in vitro* culture at temperature $24^oC \pm 20^oC$, with 16 h/8h photoperiod and phot on flux density at 1000 lux.

Data analysis

The experiment was arranged in completely randomized design with ten replications. Data obtained were analyzed by using Statistical Analysis System (SAS) portable version 9.1. Further analysis was carried out by using Duncan Multiple Range Test (DMRT) at 5% of level of significant.

RESULTS AND DISCUSSION

Shoot multiplication

Application of silver nitrate at various concentrations affected the growth of St. John's wort *in vitro*. Without application of silver nitrate, culture tend to be rosette on MS + 0.1 mgl^{-1} BA as indicated in Figure 1a. Combination of silver nitrate from low concentrations (0.1 -0.5 mgl^{-1}) with BAP could improve the growth of St John's wort which is characterized by changes of leaf size as presented in Figures 1b, c and d. It indicated that culture has more normal morphological characteristics when cultured on MS + BA 0.1 mgl^{-1} in combination with 0.1-0.5 mgl^{-1} $AgNO_3$. Increasing concentration of $AgNO_3$ to 0.7 $mg/^{-1}$ showed the signs of vitrification of the culture as shown in Figure 1e. The best treatment to obtain the normal growth was reached on MS containing 0.1 mgl^{-1} BA + 0.3 mgl^{-1} AgNO3 as indicated in Tables 1 and 2 and Figure 1c.

Rooting induction

Rooting induction on St. John's wort *in vitro* showed a low response. Application of macronutrient at a half-strength concentration, in combination with silver nitrate at 0.3 mgl^{-1} were able to produce a rooted culture even though only small percentage (30%) and the roots were still very limited as indicated in Table 3 and Figure 1f.

DISCUSSION

Addition of silver nitrate into culture media could improve the quality of culture growth by inhibiting of ethylene activity in the culture. St. John's wort culture treated with a combination of 0.1 mgl^{-1} BAP + 0.3 mgl^{-1} AgNO3

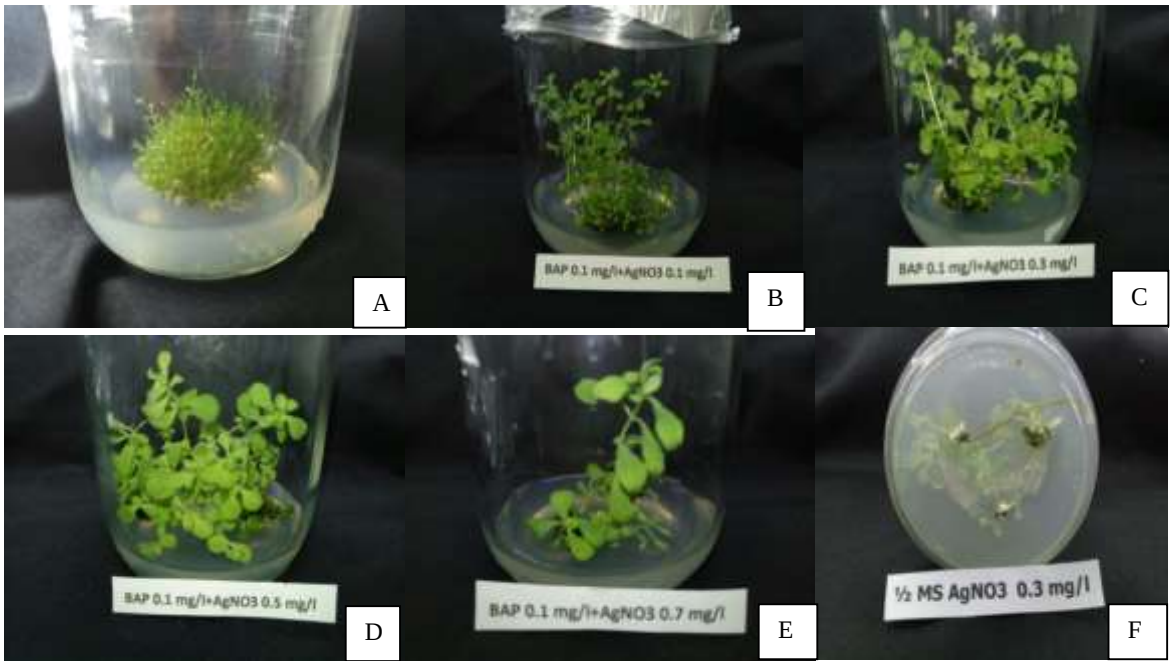

Figure 1. (A) Characteristic of St. John's wort on MS + 0.1 mgl^{-1} BA , effect of BA and $AgNO_3$ combined with several concentration levels on shoots multiplication and visual characteristics of St. John's wort *in vitro*: (B) 0.1 mgl^{-1} BA + 0.1 mgl^{-1} $AgNO_3$, (C) 0.1 mgl^{-1} BA + 0.3 mgl^{-1} $AgNO_3$, (D) 0.1 mgl^{-1} BA + 0.5 mgl^{-1} $AgNO_3$, and (E) 0.1 mgl^{-} BA 0.1 mgl^{-1} + $AgNO_3$ 0.7 mgl^{-1}. (F) Rooting induction of St. John's wort *in vitro* at ½ MS + 0.3 mgl-1 $AgNO_3$.

Table 1. Effect of combined concentration levels of BA and $AgNO_3$ on the growth of St John's wort *in vitro* at two months after culture.

Treatment (mgl^{-1})	Number of shoot	Shoot length (cm)
0.1 BA + 0.0 $AgNO_3$	9.0^{b}	8.40^{bc}
0.1 BA + 0.1 $AgNO_3$	9.6^{b}	9.00^{b}
0.1 BA + 0.3 $AgNO_3$	12.6^{a}	10.6^{a}
0.1 BA + 0.5 $AgNO_3$	8.4^{b}	8.8^{b}
0.1 BA + 0.7 $AgNO_3$	4.2^{c}	7.4^{c}

The numbers followed by the same letter in each column are notsignificantly different at 5% DMRT.

Table 2. Effect of combined concentration levels of BAP and $AgNO_3$ on plantlets characteristics of St. John's wort *in vitro* at two months after culture.

Treatment (mgl^{-1})	Plantlet characteristic
0.1 BAP + 0.1 $AgNO_3$ (control)	Leaves rather small and tended to rosette
0.1 BAP + 0.1 $AgNO_3$	Leaves were slightly larger than control
0.1 BAP + 0.3 $AgNO_3$	Leaves were normal
0.1 BAP + 0.5 $AgNO_3$	Leaves were rather large but tended to show vitrification symptom
0.1 BAP + 0.7 $AgNO_3$	Leaves showed signs of vitrification

showed the best growth performance compared to the others. However, higher concentration of silver nitrate may cause vitrification of St. John's wort culture. Plantlets having vitrification symptom usually produces larger leaves which contain much water and easy to be withered. Silver nitrate is known to promote multiple

Table 3. Effect of various levels of silver nitrate concentration on rooting induction of St. John's wort *in vitro.*

Treatment (mgl^{-1})	Rooting percentage (%)	Number of root	Root length (cm)
½ MS + 0.1 $AgNO_3$	0.0^b	0.0^b	0.0^b
½ MS + 0.3 $AgNO_3$	30.2^a	1.3^a	3.3^a
½ MS + 0.5 $AgNO_3$	1.9^b	0.7^b	1.2^b
½ MS + 1.0 $AgNO_3$	0.0^b	0.0^b	0.0^b

The numbers followed by the same letter in each column are not significantly different at 5% DMRT.

shoot formation in different plants. *In vitro* shoot formation was improved by incorporating silver nitrate in the culture medium.

Most plants that are propagated through tissue culture produced ethylene which may affect the growth of culture. The influence of ethylene on the growth of culture was greatly varied depending on plant sensitivity. Silver nitrate is usually applied to inhibit ethylene activity in *in vitro* propagation. Silver ions are able to prevent a variety of responses displayed by plants due to the influence of ethylene (Beyer et al., 1984).

The exact mechanism of $AgNO_3$ action on plants is unclear; however, few existing evidences suggest its interference in ethylene perception mechanism (Beyer, 1976c). In recent years, $AgNO_3$ has been employed in tissue culture studies for inhibiting ethylene action because of its water solubility and lack of phytotoxicity at effective concentrations (Beyer, 1976a). In *S. nigrum* culture, the use of silver nitrate is able to increase shoot multiplication, the same as can be observed in *Coffea arabica* and *V. planifolia* plants (Ganesh and Sreenath, 2008; Sankar et al., 2008). The addition of different concentrations of $AgNO_3$ (10, 30 and 50 M) to the medium, however, induced shoot regeneration in distal cotyledon except Suyo Long cultivar and effectively increased shoot regeneration response as well as the number of shoots per explant in proximal cotyledon and hypocotyl of all cucumber cultivars (Mohiuddin et al., 1997).

Results of preliminary research showed that the use of macronutrients at full concentration with the addition of auxin IBA has not been able to induce rooting perfectly (Syahid, 2008). Response on silver nitrate is varying in different plants. In *S. nigrum* culture, a combination of IBA 2.0 mgl^{-1} with silver nitrate 0.4 mgl^{-1} was able to produce roots up to 24.6 ± 0.26 (Geetha et al., 2016). The effect of $AgNO_3$ on rooting and shooting was evaluated in *V. planifolia*. Application of silver nitrate into the medium showed positive response not only on shoot initiation, number and growth, but also increased root number and length. Maximum number of shoots and highest shoot length was obtained on medium containing 20 µM $AgNO_3$. Application of $AgNO_3$ not only induced shoot multiplication but also influenced rooting of vanilla explants (Giridhar et al., 2001).

Conclusion

Application of silver nitrate in combination with BA was able to improve the growth of St. John's wort (*Hypericum perforatum) in vitro*. The combination of silver nitrate at concentration of 0.3 mgl^{-1} with 0.1 mgl^{-1} BA produced the highest number of shoots, longest shoot and largest quantity of leaves (10.6 leaves) within two months. Silver nitrate was able to induce normal morphological characteristics of St. John's wort which was indicated by normal stems and leaves growth during culture period. The best rooting induction was obtained on ½ MS + 0.3 mgl^{-1} $AgNO_3$.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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