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RESEARCH ARTICLE

Tissue culture protocol establishment of *Artemisia annua* L. plant and artemisinin production

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Abstract

In vitro culture parameters were investigated in order to develop an effective protocol for *Artemisia annua* cultivation and artemisinin production. The explants, shoot tips and axillary buds, were individually cultured on MS medium contained 6-benzylaminopurine (BAP) or growth regulators. Treatment with mercuric chloride (1.0%) for 5 min gave the highest survival percentage (86.0% and 90.0%) and the lowest contamination percentage (40.0% and 64.0%) for terminal shoot tip and axillary bud, respectively. Axillary bud explants surpassed shoot tips in development and direct regeneration. High concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA) plus 0.5 mg/L BAP were suitable for callus induction while low concentrations and the control induced less necrosis and more explant development, as well as increased artemisinin concentration. Lower BAP concentrations induced an increased growth rate, while the higher BAP concentration encouraged proliferation. Seven treatments with different concentrations of NAA, 2,4-D, and salicylic acid produced lower amounts of artemisinin than that produced by control (0.5 mg/L BAP). Decreasing level of artemisinin could be due to reduced rates of plant growth and decreased amounts of green matter by growth regulators, which may affect the plastidic pathway for artemisinin production.

Keywords: *Artemisia annua*; Callus; Direct regeneration; Plant growth regulators

1. Introduction

Medicinal plants are considered as an important therapeutic source for a variety of human illnesses (Dev, 1997). Given the importance of medicinal plants, there are several studies to improve the productivity of such plants and their active substances under different conditions (Hendawy *et al*., 2017, 2019). *Artemisia annua* L. belongs to the family of Asteraceae and produces aromatic oils and essential substances. The major active substance is artemisinin, which has anti-plasmodium properties (Klayman, 1985; Sy and Brown, 2001). The complexity of synthesizing artemisinin reduces its economic competitiveness with naturally occurring artemisinin (Xu *et al*., 1986). As a result, *in vitro* artemisinin synthesis is an excellent technique to ensure year-round production of this crucial molecule. Recently, artesunate which is derived from artemisinin revealed as an effective treatment for COVID-19 (Uzun and Toptas, 2020).

When it relates to artemisia (*Artemisia annua* L.) shoot induction, cultured nodal explants exceed shoot tip explants (Hailu *et al*., 2013). Shoot regeneration of *A. absinthium* can be induced on half-strength MS medium (Mannan *et al*., 2012). Although using half-strength MS media is more suitable for germination of *A. annua*, full strength MS media enable seedlings to be transferred two weeks after germination (Mohammad *et al*., 2014). When comparing calluses induction from nodal explants of *A. annua* after adding different growth regulators to media culture (2.5, 5.0, 7.5, or 10 µM NAA, 2,4-D, indole-3-butyric acid (IBA) and indole acetic acid (IAA), the best result of callus induction (about 90 %) was found

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with MS medium supplemented with 2.5 µM of IAA (Shinede *et al*., 2016). Combinations of IBA and NAA produced compact calluses in the leaf explant, although they varied in color and growth pattern. The dark brown, greenish, and green-colored calluses grew well under light conditions on modified MS supplemented with a combination of 6-benzyl aminopurine (BAP) (0.1 mg/L) and NAA (0.5–2.0 mg/L) (Mohammad *et al*., 2014). In addition, the culture medium supplemented with 2.5 µM IAA induced highly proliferating organogenic callus of *A. nilagirica* (Indian worm wood). High regeneration capacity (83.3%) of adventitious shoots was also observed when the callus was cultured on MS medium contain 2.5 µM of 6-benzylaminopurime and 7.5 µm 2-isopentenyl adenine. The highest number of shoots appeared on medium supplemented with 2.5 µM BAP and 7.5 µM isopentenyl adenine (2iP) (Shinede *et al*., 2016). Similarly, supplementation with BAP at 0.5 mg/L gave the best development of shoots multiple from nodal explants for Wolfberry plant (Silvestri *et al*., 2018).

For the transformation of farnesyl diphosphate (FPP) to artemisinin, there are two pathways. The first is the cytoplasmic mevalonate (MVA) pathway, which employs two isoprenoid precursors derived from acetyl-CoA: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The second is a plastidic non-MVA pathway where IPP is produced from glyceric acid-3-phosphate and pyruvate. IPP produced from the mevalonate pathway produces FPP, whereas the non-mevalonate route produces geranyl diphosphate (GPP), which moves to the cytosol to produce FPP and ultimately artemisinin (Schramek *et al*., 2010).

The aim of this study was to investigate the effect of explants, plant growth regulators, sterilization methods and MS medium strength on *in vitro* proliferation and artemisinin production in *Artemisia annua* plant.

2. Materials and Methods

2.1. Materials

Axillary buds and shoot tips were selected from plants of *Artemisia annua* L. that have been grown in the field or greenhouse, National Research Centre, Giza, Egypt. The selected explants were washed with soap solution and then cultivated in the laboratory under aseptic conditions. Sterilization of explants was carried out with 0.1% of $HgCl_2$ (w/v) for five minutes before being rinsed several times with distilled sterile water.

Sterilized explants were cultured on MS basal medium contain 30g/L sucrose, 0.5mg/L BAP (6-benzyl aminopurine) and 7g/L DifcoBactoagar, and pH was 5.7. Media was autoclaved at 121°C and 151 lb/in^2 for 15 minutes. All cultures were incubated at 27°C with a 16 h photoperiod of 2000–4000 lux.

2.2. Experimental

2.2.1. Surface sterilization

Collected explants were sterilized using a solution of commercial bleach 0.525% and 1.05% sodium hypochlorite (NaOCl) for 10 min. The other sterilizing agent was mercuric chloride ($HgCl_2$) at 0.1% and 1.0% for 5 min. Survival and contamination percentages were recorded four weeks after culturing.

2.2.2. Effect of explant type

Shoot tip and axillary bud explants were tested to select the best type of cultured explant inducing the most effective explant development.

2.2.3. Effect of medium strength

Full, one-half, and one-quarter MS strengths were tested to determine the best medium strength for improving growth metrics.

2.2.4. Effect of growth regulators

NAA, Salicylic acid and 2,4-D were added to MS medium with concentrations of 1.0 and 2.0 mg/L to mark the most effective concentration for inducing shoot regeneration.

2.2.5. Effect of different concentration of BAP

BAP was tested at 0.5, 1.0, 1.5, and 2.0 mg/L to mark the most suitable concentration that induce the highest proliferation.

2.2.6. Determination of the artemisinin content

Ferreira *et al*. (1994) described the method of artemisinin extraction. Dry leaves (0.5 g) were soaked in 50 mL of hexane at 75ºC for 1 hr. The hexane solution allowed to evaporate to dryness and the samples were dissolved in 10 mL of acetonitrile, then filtered through nylon Millex-GN filters. Filtered aliquots samples were analyzed by high-performance liquid chromatography (HPLC) (Peng *et al*., 2006).

2.2.7. Data and calculations

Scores were given for necrosis, explant development, callus production, plantlets regeneration and greening according to (Pottino, 1981). The experimental treatments were performed in a complete randomized block design and replicated five times with explants for each replicate. Data was assessed by analysis of variance and statistically analyzed according to Duncan's test (Duncan, 1955).

3. Results and Discussion

3.1. Effect of Surface sterilization on survival and contamination percentage

Table (1) showed the survival and contamination percentage of *Artemisia annua* L. after surface sterilizing with NaOCl and $HgCl_2$. The terminal shoot tip and axillary bud survived well when exposed to 0.1 percent $HgCl_2$ for 3 minutes (86.0 % and 90.0 %, respectively). However, treatment with 0.525% NaOCl for 10 mins resulted in the lowest percentage survival (43.0% and 50.0%) for terminal shoot tip and axillary bud, respectively, as compared with other treatment conditions.

For contamination percentage, 0.525% NaOCl for 10 min resulted in the highest contamination percentage for terminal shoot tip and axillary bud explant (80.0% and 88.0%, respectively) followed by 1.05% NaOCl for 10 min (63.0% and 67.0%, respectively). Treatment with 1.0% $HgCl_2$ for 3 min led to lower contamination percentage (40.0% and 64.0%, respectively). Therefore, using 1% $HgCl_2$ for both *A. annua* explant types (shoot tip and axillary bud) by dipping for three min duration gave high survival percentage and low contamination percentage compared with results from other treatments.

3.2. Effect of explant type on explant development

Callus production and direct regeneration parameters were shown in Table (2). Use of axillary bud explants resulted in a highly significant increase in explant development and direct regeneration compared with that produced from shoot tips. However, there was no significant difference between the explant material when necrosis and callus production were considered. Generally, axillary bud surpassed shoot tips in explant development and direct regeneration.

Table 1. Effect of surface sterilization with sodium hypochlorite (NaOCl) and mercuric chloride ($HgCl_2$) on survival and contamination percentages of *Artemisia annua*L.*in vitro* culture.

Surface sterilization	Explant survival %		Contamination%	
Reagent %	Shoot tip [a]	Axillary bud	Shoot tip	Axillary bud
NaOCl 0.525%	43.00±0.58^{d}	50.00± 0.58^{d}	80.00±0.58^{a}	88.00± 0.58^{a}
NaOCl 1.05%	65.00 ± 0.58^{c}	70.00±0.58^{c}	63.00±0.58^{b}	67.00± 0.58^{b}
$HgCl_2$ 0.1%	73.66± 1.73^{b}	85.00±1.15^{b}	55.00±0.58^{d}	53.00± 0.58^{c}
$HgCl_2$ 1.0%	86.00 ±0.58^{a}	90.00±1.15^{a}	40.00±0.58^{c}	46.00± 0.58^{d}
LSD5%	2.997	3.313	2.663	1.998

a the same letter(s) revealed that the values are not significantly different (Duncan test, P=0.05).

Table 2. Effect of explant type on explant development, callus production and direct regeneration parameters of *Artemisia annua*.

Explant type	Growth parameters			
	Necrosis[a]	Explant development	Callus production	Direct regeneration
Shoot tip	1.00±0.06^{b}	2.67±0.01^{b}	2.00±0.06^{b}	3.00±0.06^{b}
Axillary bud	1.30±0.06^{b}	3.67± 0.01^{a}	2.00±0.12^{b}	4.00±0.12^{a}
LSD5%	2.484	0.043	ns	0.745

a the same letter(s) revealed that the values are not significantly different (Duncan test, P=0.05).

3.3. Effect of medium strength on explant development

Callus production and direct regeneration parameters of *A. annua* were shown in Table (3). Full and one-half medium strengths significantly increased explant development as compared with that achieved using one–quarter median strength. Direct regeneration was significantly increased on one-half strength of MS medium followed by that produced on full strength as compared with that from one-quarter strength. However, there was no significant difference in necrosis or callus production parameters among the different medium strengths used. Therefore, we concluded that full and one-half strengths of MS medium improved explant development and direct regeneration. This may be due to the high nutrient concentration required for *A. annua*.

3.4. Effect of BAP concentration

The effect of different concentrations of BAP on *Artemisia annua* growth and proliferation is described in Table (4). Both growth and greening were significantly increased when a lower BAP concentration (0.5mg/L) was used as compared with higher concentrations (1.5 and 2.0 mg/L). Treatment with 2.0 mg/mL BAP resulted in a significant increase in proliferation compared with that induced by control, which was not evident with other concentrations used. However, increasing the concentration of BAP to 2.0 mg/L also significantly increased necrosis, whereas lower concentrations (0.5 and 1.0 mg/L) generally resulted in reduced necrosis and improved both growth and greening.

3.5. Effect of growth regulators on explant development and callus production

The growth regulators effects on explant development and callus production parameters of *A. annua* were shown in Figures (1 and 2) and

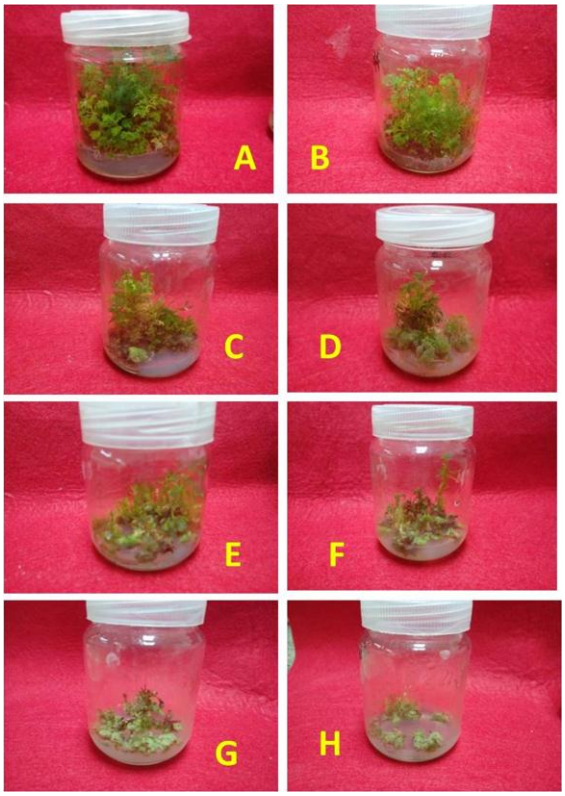

Figure 1. Effect of growth regulators on explant development and callus production parameters of *Artemisia annua* L. A, control 0.5mg/L BAP; B,1.0 mg/Lsalicylic acid +0.5 mg/L BAP; C, 1.0 mg/LNAA +0.5mg/L BAP;D,2.0 mg/L NAA+0.5mg/L BAP;E,4.0mg/LNAA +0.5 mg/L BAP;F,1mg/L 2,4-D +0.5 mg/L BAP;G, 2mg/L 2,4-D+0.5 mg/L BAP;H, 4mg/L 2,4-D +0.5 mg/L BAP

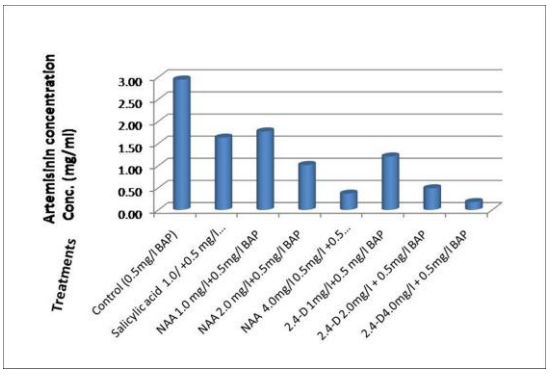

Figure 2. Effect of growth regulators on artemisinin concentrations in *in vitro* culture of *Artemisia annua*. plant.

Table 3. Effect of different medium strength on explant development, callus production, and direct regeneration parameters of *Artemisia annua*.

MS Medium strength	Necrosis[a]	Explant development	Callus production	Direct regeneration
Full	1.00±0.56[b]	4.67±0.03[a]	1.00±0.06[a]	3.33±0.19[b]
One-half	1.33±0.13[a]	4.33±0.19[b]	1.00±0.12[a]	4.67±0.02[a]
One-quarter	1.33±0.02[a]	2.30±0.06[c]	1.00±0.17[a]	2.00±0.12[c]
LSD5%	0.236	0.338	ns	0.329

a: the same letter(s) revealed that the values are not significantly different (Duncan test, P=0.05).

Table (5). Under control conditions (0.5 mg/L BAP), the lowest level of necrosis together with significantly increased development of explants were produced followed by low concentrations of NAA or 2,4-D (1mg/L) combined with BAP (0.5 mg/L), and to a lesser extent by treatment with salicylic acid (1 mg/L) combined with BAP (0.5 mg/mL) compared with effects produced by other treatments used. In addition, 2, 4-D (2mg/L) combined with BAP (0.5 mg/L) had the most significant effect on callus production followed by high concentrations of NAA or salicylic acid. The combination of BAP with low concentrations of NAA or IBA in medium has been previously found to be effective in increasing explant development of *A. annua*.

3.6. Effect of growth regulators on artemisinin content

The effects of growth regulators on the artemisinin content of *A. annua* grown on MS medium were shown in Figures (3–10). The control (0.5 mg/L BAP) produced high concentrations of artemisinin compared with treatment with salicylic acid, NAA, and 2,4-D. In contrast, artemisinin accumulation decreased with increased concentrations of salicylic acid, NAA, and 2,4-D. There was a clear significantly increase in artemisinin concentration with the use of salicylic acid (1mg/L) combined with BAP (0.5 mg/L) and under the low concentration of NAA or 2,4-D (1 mg/L) combined with BAP (0.5mg/L) as compared with the results from the other treatments. Followed by, NAA or 2,4-D (2mg/L) combined with 0.5mg/L BAP. The lowest artemisinin concentration was produced by the high concentrations of NAA or 2, 4-D (4mg/L) combined with BAP (0.5 mg/L). Decreases in artemisinin concentration may be correlated with decreasing plant biomass and/or chlorophyll and/or down regulation of gene expression involved in artemisinin pathway production, which are affected by NAA, 2, 4-D, or salicylic acid.

4. Discussion

Contamination by microorganisms, either in the used explants or in the surrounding environment, is one of the obstacles encountered during tissue culture establishment. The percentages of contaminations that appeared in the media, on the other hand, varied depending on the types of explants employed and the treatments used. Whereas the highest contamination percentage for terminal shoot tip and axillary bud explant were 80.0% and 88.0%, respectively, after

Table 4. Effect of BAP concentrations of MS medium on growth and proliferation of *Artemisia annua*.

Growth parameters / BAP con. (mg/L)	Necrosis [a]	Growth	Proliferation	Greening
0.50	1.00±0.06^{c}	3.27±0.03^{a}	2.67±0.03^{b}	4.67±0.03^{a}
1.00	1.00±0.12^{c}	2.33±0.13^{b}	2.33±0.06^{b}	3.67±0.03^{b}
1.50	2.33±0.10^{b}	2.00±0.012^{c}	2.33±0.19^{b}	2.33±0.13^{c}
2.00	4.16±0.02^{a}	1.67±0.03^{d}	4.30±0.12^{a}	2.00±0.12^{d}
LSD5%	0.304	0.185	0.422	0.185

a the same letter(s) revealed that the values are not significantly different (Duncan test, P=0.05).

Table 5. Effect of growth regulators on explant development and callus production parameters of *Artemisia annua*.

Growth regulator treatment	Necrosis[a]	Explant development	Callus production
BAP (0.5 mg/LBAP) Control	1.00±0.06^{e}	4.00±0.12^{a}	1.00±0.06^{e}
Salicylic acid (1.0 mg/L +0.5 mg/L BAP)	1.30±0.12^{d}	3.67±0.03^{b}	1.67±0.03^{d}
NAA (1.0 mg/L +0.5mg/L BAP)	1.00±0.12^{e}	3.00±0.06^{c}	2.00±0.12^{d}
NAA (2.0 mg/L +0.5mg/L BAP)	2.00±0.12^{c}	2.00±0.06^{d}	4.00±0.29^{b}
NAA (4.0mg/L +0.5 mg/L BAP)	3.00±0.06^{b}	2.00±0.12^{d}	4.00±0.06^{b}
2,4-D (1.0 mg/L +0.5 mg/L BAP)	1.00±0.06^{e}	3.00±0.17^{c}	3.00 ±0.06^{b}
2,4-D (2.0mg/L + 0.5mg/L BAP)	3.20±0.15^{b}	1.67 ±0.03^{e}	4.67±0.03^{a}
2,4-D (4.0mg/L + 0.5mg/L BAP)	3.80 ±0.06^{a}	2.80±0.06^{c}	4.37 ±0.09^{ab}
LSD5%	0.264	0.273	0.375

a: the same letter(s) revealed that the values are not significantly different (Duncan test, P=0.05).

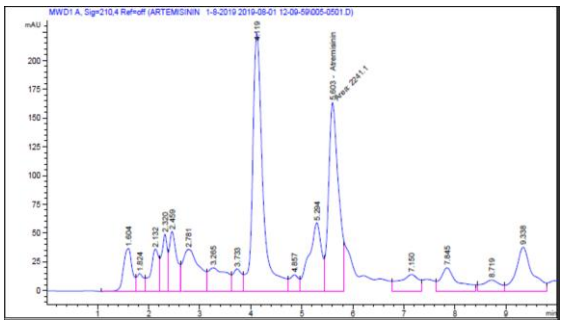

Figure 3. Artemisinin production detection under control (0.5mg/L BAP).

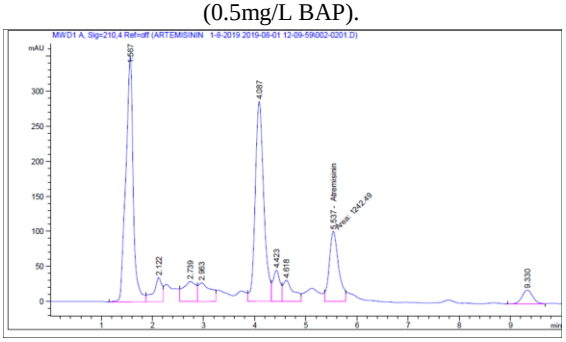

Figure 4. Effect of salicylic acid (1.0 mg/L) +BAP (0.5 mg/L) treatment on artemisinin production

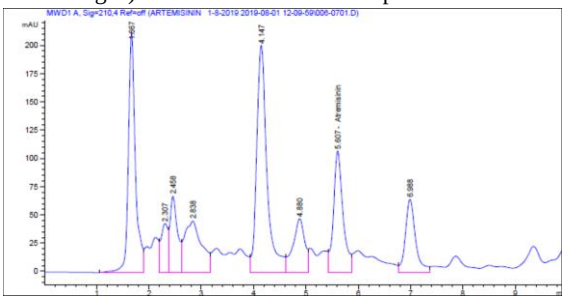

Figure 5. Effect of NAA (1.0 mg/L) +BAP (0.5 mg/L) treatment on artemisinin production

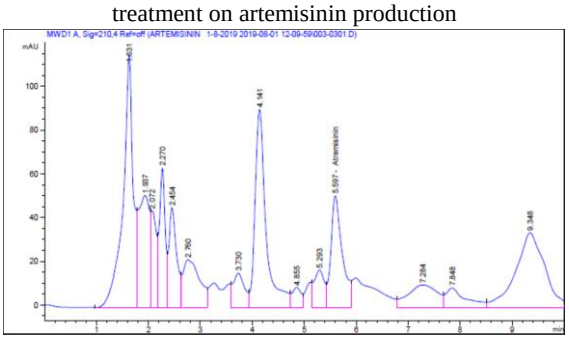

Figure 6. Effect of NAA (2.0 mg/L) +BAP(0.5mg/L) treatment on artemisinin production.

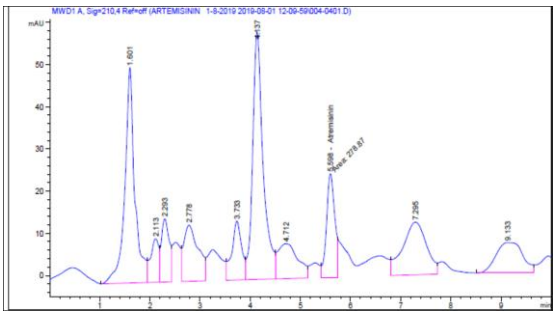

Figure 7. Effect of NAA (4.0 mg/L) + BAP(0.5mg/L) treatment on artemisinin production.

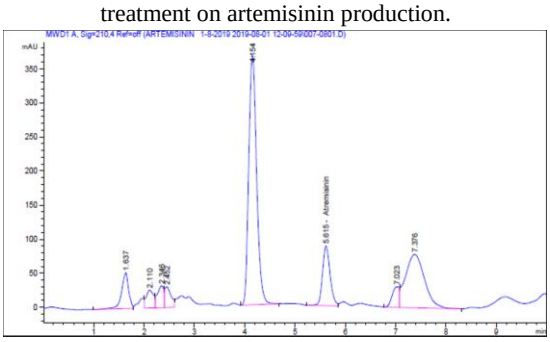

Figure 8. Effect of 2,4-D (1mg/L) + BAP (0.5 mg/ L) treatment on artemisinin production.

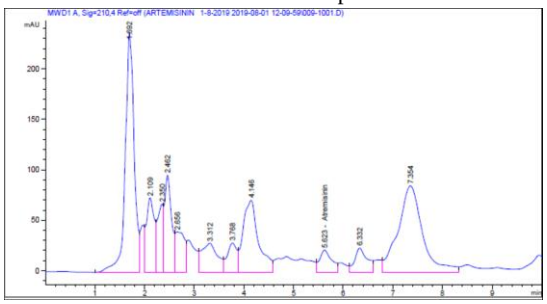

Figure 9. Effect of 2,4-D (2.0mg/L) +BAP (0.5mg/L) treatment on artemisinin production.

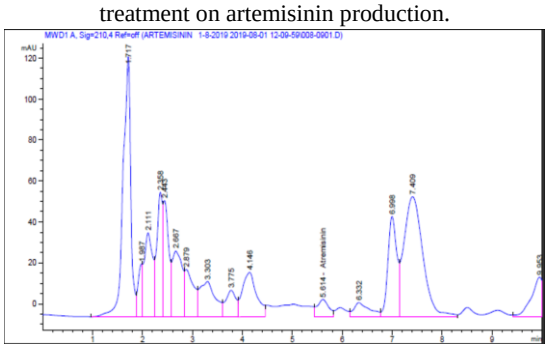

Figure 10. Effect of 2,4-D (4.0mg/L) +BAP (0.5mg/L) treatment on artemisinin production.

treatments with 0.525% NaOCl for 10 min. This percentage decreased in followed by 1.05% NaOCl for 10 min for terminal shoot tip and axillary bud to be 63.0% and 67.0%, respectively. On the other hand, the lowest contamination percentage for shoot tips and axillary buds with a high survival percentage by treatment with 1.0% $HgCl_2$ for 3 min. These results exhibited the strength of $HgCl_2$ compared with sodium hypochlorite for facing microorganisms that in turn decreasing the contamination (Al maarri and Xie, 2010). This result is in accordance also with (Xu *et al*., 2005) who found that $HgCl_2$ was better than NaOCl for surface sterilization of *Pinellia ternata*. Sterilization with 0.1% $HgCl_2$ for 4 min was effective for Strawberry explants *in vitro* culture and the treatment revealed a maximum

percentage of explants survival (Jan *et al*., 2013). However, our results were not in agree with (Badoni and Chauhan, 2010) who showed that the best sterilizing agent was NaOCl in potato sprout treatment. Whereas, both sterilization agents; NaOCl (1%) and $HgCl_2$ (0.1%) were used at three exposing times; 2, 5 and 8 minutes, and found that NaOCl for 8 minutes was better than other treatments for sterilization to control the infection and did not show any effect on explants survival in long duration. Moreover, both sterilizing agents, NaOCl and $HgCl_2$, were effective in cleaning Kinnow tree explants (Nakamura, 1991; Altaf, 2006). On the other hand, the lowest contamination percentage appeared in axillary buds compared to shoot tips with a high percentage survival especially under treatment with $HgCl_2$. The increased multiplication rate of axillary buds relative to shoot tips appeared to be increasing survival percentage, especially at the beginning of transplanting. This result is similar to what was discovered with the Musa cultivar. (Youmbi *et al*., 2014) who reported that no significant differences were observed between the two types of buds {axillary buds and shoot tips (apical buds)} after four to five subcultures in all the varieties except for CRBP 39 where the axillary bud showed a higher proliferation rate. Substantiation our interpretation of the above results, the effect of explant type on explant development, callus production and direct regeneration parameters were studied. When axillary bud explants were used instead of shoot tips, there was a considerable increase in explant development and direct reproduction. These results are consistent with the findings of (Hailu *et al*., 2013; Gopinath *et al*., 2014), whom found the best growth of *A. annua* explants occurred from axillary buds compared to that from shoot tip explants. In addition, explant development, regeneration, proliferation, and callus production depend on the components of MS medium. In our study, we present an efficient protocol for high-frequency regeneration from *A. annua*.

Other mechanisms that could lead to rapid multiplication and huge production are required to develop some therapeutic plant compounds that have a poor reproductive potential but high value. Furthermore, indirect organogenesis can be used to improve plant genetics, preserve germplasm, and produce beneficial secondary metabolites (Hesami and Daneshvar, 2018). In this study, we also examined the effects of some plant growth regulators and their concentrations on the formation of each of these pathways to establish prolific and rapid *in vitro* axillary buds and shoot tip explants for detecting the effect of different strengths of MS medium on explant development, callus production and direct regeneration parameters of *A. annua*. Full and one-half medium strengths were used and they showed significantly impacts on explant development as compared with that achieved using one–quarter median strength. Direct regeneration was significantly increased on one-half medium strength followed by that produced on full medium strength as compared with that from one-quarter medium strength. Therefore, it is concluded that full and one-half medium strengths improved explant development and direct regeneration. This may be due to the high nutrient concentration required for *A. annua*. and agrees with previous findings that reported half-strength of MS medium is more suitable for shoot regeneration of *A. annua* (Hailu *et al*., 2013; Mannan *et al*., 2012; Mohammad *et al*., 2014; Gichana *et al*. 2019).

Plant growth regulations play a necessary role in *in vitro* organogenesis such as callus induction and shoots regeneration (Hesami and Daneshvar, 2018; Zhao, 2008). The desired plant growth regulation concentrations in plant tissue culture differ from species to species, while the cytokinin/auxin ratio plays an important role in *in vitro* culture (Fukai and Oe, 1986; Jones *et al*., 2010; Gichana *et al*., 2019). Growth regulators effect on explant development and callus production parameters of *A. annua* was studied. The lowest level of necrosis together with significantly increased development of explants were produced under control, followed by low concentrations of NAA or 2,4-D, and salicylic acid compared to other higher treatments. On the

other hand, high concentrations of 2,4-D showed significant effect on callus production followed by high concentrations of NAA or salicylic acid. The combination of BAP with low concentrations of NAA or IBA in medium has been previously found to be effective in increasing explant development of *A. annua* (Shinede *et al*., 2016; Uzun and Toptas, 2020) whom reported that callus induction from nodal explants of *A. annua* on medium contain 2.5, 5.0, 7.5, and 10.0 µM NAA, 2,4-D, and IBA. Cytokinin BAP has a key role in plant development during formation and activity of shoot meristems (Kyozuka, 2007). BAP enhances proliferative rates in various plant species and is involved in the shoot proliferation improvement and elongation (Glocke *et al*., 2006). In the present investigation, we studied the effects of various concentrations of BAP on growth and proliferation of artemisia. The results showed that both growth and greening were significantly increased when a lower concentration of BAP was used as compared with higher concentrations. Treatment with higher concentration of BAP resulted in a significant increase in proliferation compared with that induced by control, which was not evident with other concentrations used (Han *et al*. 2005; Hailu *et al*., 2013; Gopinath *et al*. 2014; Shinede *et al*., 2016). The higher concentration of BAP also significantly increased necrosis, whereas lower concentrations generally resulted in reduced necrosis and improved both growth and greening. Furthermore, we found that a higher multiplication rate of *A. annua* was obtained with supplementation of MS medium using high concentration of BAP (Muhammad *et al*., 2007). Moreover, the control (0.5 mg/L BAP) produced high concentrations of artemisinin compared with treatment with salicylic acid, NAA, and 2,4-D. In contrast, artemisinin accumulation decreased with increased concentrations of salicylic acid, NAA, and 2,4-D. There was a clear significantly increases in artemisinin concentration with the use of salicylic acid (1mg/L) combined with BAP (0.5 mg/L) and the low concentration of NAA or 2,4-D (1mg/L) combined with BAP (0.5mg/L) as compared with other treatments. Decreases in artemisinin concentration may correlate with decreasing plant biomass and/or chlorophyll and/or down regulation of gene expression involved in artemisinin pathway production, which are affected by NAA, 2, 4-D, or salicylic acid. In previous studies, NAA has in general inhibitory effect on the carotenoid and chlorophyll content in leaves (Czerpak *et al*., 2002). Salicylic acid treatment of barley seedlings resulted in poor rates of growth and photosynthesis, which has been linked to changes in the anatomy and ultrastructure of chloroplasts (Uzunova and Popova, 2000). Moreover, treatment of wheat plants with salicylic acid led to decreases in both carotenoid and chlorophyll contents (Moharekar *et al*., 2003). Treatment with 2,4-D also reduced plant size and chlorophyll contents of maize plants (Gomes *et al*., 2017).

5. Conclusion

In this study, a protocol has been developed for Artemisia culture and production of the anti-malarial plant metabolite, artemisinin. *Artemisia annua* axillary bud and shoot tip explants were cultured individually on MS medium contain BAP or different plant growth regulators. Axillary bud explant surpassed shoot tip in explant development and direct regeneration, whereas there was no significant effect on necrosis and direct regeneration. Additionally, high concentrations of NAA and 2,4-D with BAP were appropriate for callus induction, while low concentrations and the control induced less necrosis and increased explant development parameters including artemisinin concentration. The lower BAP concentration induced a low level of necrosis and a high growth rate, whereas higher BAP concentrations encouraged proliferation. Treatments with various concentrations of NAA, salicylic acid and 2,4-Dproduced lower amounts of artemisinin in *in vitro* culture of *A. annua* plant compared with that of control.

6. References

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