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# Optimizing Hairy Root Culture Media Using Salts and Vitamin Modifications in *Corylus avellana* L.

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A R T I C L E I N F O

*Keywords:*

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A B S T R A C T

Paclitaxel (taxol) has been recognized as a very effective anti-cancer drug. Today, hazelnuts, as one of the natural sources of this medicinal compound, have been heeded more than before. Optimizing culture conditions to promote hairy root growth is an important step towards the production of medicinal metabolites. In this study, we developed an efficient culture medium to increase the production of hazelnut hairy roots. To achieve this aim, different intensities (full, $\frac{1}{2}$ and $\frac{1}{4}$) of WPM, SH, NRM, and DKW media, and the replacement of vitamins with the B5 medium vitamins were evaluated. We found that $\frac{1}{4}$ WPM + vit B5 increased hairy roots growth in solid nursery medium and $\frac{1}{4}$ SH + vit B5 was the effective media for hairy roots development in liquid culture media. Appling 100 mg $l^{-1}$ ascorbic acid and citric acid reduced the browning of hairy roots and improved their growth rate. The paclitaxel production in hairy roots was assured by using HPLC.

**Abbreviation AA** Ascorbic Acid, **CA** Citric Acid, **DKW** Driver Kuniyuki, **DW** Dry Weight, **PC** Paclitaxel, **WPM** Woody Plant Medium, **NRM** Nas and Read Medium, **SH** Schenk and Hildebrandt medium

## Introduction

Paclitaxel©(PC) is a diterpenoid plant metabolite with potent anti-mitotic action and accordingly, with biological anti-cancer activities (Kohler and Goldspiel, 1994; Schiff *et al.*, 1979). *Taxus* spp. has been the first and main source of PC. The major limitation is its massive demands and low supply of this compound due to the low PC content in *Taxus* spp. Direct extraction of PC from the bark of *Taxus* has exposed this tree to the risk of extinction. The biotechnological approaches, such as cell suspension culture and hairy root culture are promising methods to eliminate these limitations in PC production (Bestoso *et al.*, 2006). In addition, *Taxus* spp., hazelnut (*Corylus avellana)* has also been defined as the only angiosperm which can produce PC (Hoffman, 1998; Service, 2000). However, the PC contents in hazelnut tissues are lower than their contents in *yew*, hazelnut is an easily accessible plant, and its *in vitro* culture is more reliable than yew (Bestoso *et al.*, 2006; Gallego *et al.*, 2015). Recently, several studies have also disseminated hazel cell culture as a new source of PC (Rahpeyma *et al.*, 2015; Salehi *et al.*, 2017; Salehi *et al.*, 2019a). Hazelnut cell cultures are potent in modifying PC content (Rahpeyma *et al.*, 2017;

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Salehi *et al.*, 2019a). Besides, hazelnut is considered as a valuable source of proteins, phenolic components, vitamins, and antioxidants (Alasalvar *et al.*, 2006; Amaral *et al.*, 2005).

Since the importance of PC, attempts have been made to declare and improve its production sources (Jalalipour Parizi *et al.*, 2020; Kumar *et al.*, 2019; Salehi *et al.*, 2019b).

Transgenic hairy roots are the biotechnological platforms that have been revolutionized the production and development of plant secondary metabolites. They are unique in their genetic and biosynthetic stability, faster and hormone-independent growing, more easily maintaining and synthesizing a wide range of chemical compounds (Giri and Narasu, 2000; Shanks and Morgan, 1999). The most noticeable upside of hairy root cultures is that they often exhibit the same or higher biosynthetic capacity for secondary metabolites than their mother plants (Anthony and Davey, 2010). Hairy roots have also been considered as the efficient biological systems for commercial-scale production, clarification of biosynthetic pathways, and metabolic engineering of secondary metabolites (Sharma *et al.*, 2013). They have been introduced as a novel system for taxol production (Kim *et al.*, 2009) capable of substantial enhancement of PC content in *Taxus* hairy roots (Syklowska-Baranek *et al.*, 2009; Sykłowska-Baranek *et al.*, 2019). Additionally, the induction of PC-producing hairy roots has been reported in *C. avellana* L. by Jalalipour Parizi *et al.* (2020).

The composition of culture media and the growing condition have been found to significantly affect the success of plant tissue culture (Ashrafi et al., 2010; Naderi and Mahmoudi, 2017; Sarikhani and Sarikhani-khorami, 2021). Therefore, it is essential to make efforts to modify the culture medium composition by removing the disadvantages and improving the advantages of medium according to the plant and the aim of micropropagation in order to achieve a protocol for maximum regeneration (Ashrafi et al., 2009; Vahdati et al., 2009; Eshghi Khas et al., 2020). In the present study, the authors tried to access new modifications in culture media composition including mineral salts concentrations and vitamins, to optimize hairy roots culture in *C. avellana* L.

## Materials and Methods

### *Explant preparation and hairy roots induction*

The hazelnut hairy roots were induced in the *in-vitro* seedlings of *C. avellana* L. Hazelnut seeds had been collected from the Gilan Province of Iran (37.1378° N, 50.2836° E). In brief, the inoculation of *in-vitro* leaf and cotyledon explants with *Agrobacterium rhizogenesis* strain C58C1PRIA4 resulted in hairy roots formation of hazelnut. The first, hairy roots appeared 8 days after inoculation and continued for two months. *A. rhizogenesis*-free hairy roots nursery stocks was kept at solid $\frac{1}{4}$ WPM medium at 25°C in the darkroom. The transformation accuracy of hairy roots has been confirmed with PCR analysis, using the *rolC* gene in the TL-DNA and *virD* primers (Jalalipour Parizi *et al.*, 2020).

### *Culture media amendment experiments*

Three independent experiments were carried out based on Completely Randomized Design (CRD) with five replications. In all experiments, each replication consisted of tissue culture petri dishes (100 mm x 15 mm) or Erlenmeyer flask (with a size of 100 ml) containing 30 ml medium. The first experiment, adapted from Jalalipour Pariziz et al., (2020), was performed to assess some modified media (Table 1) to improve hairy roots growth in the solid nursery media. Different concentrations of 4 basic media formed the basis of this experiment. The effects of substitution of original medium vitamins with Gamborg (B5) medium vitamin has been evaluated on hairy roots growth, separately.

The media include WPM, $\frac{1}{2}$ WPM, $\frac{1}{4}$ WPM, SH, $\frac{1}{2}$ SH, $\frac{1}{4}$ SH, NRM, $\frac{1}{2}$ NRM, $\frac{1}{4}$ NRM, DKW, $\frac{1}{2}$ DKW, $\frac{1}{4}$ DKW and Also, $\frac{1}{4}$ WPM + vit B5 and $\frac{1}{4}$SH + vit B5. The second experiment was designed to study the effect of the above-mentioned media, by the main focus on modified vitamin on hairy roots growth and development in the liquid culture (Table 1). The third experiment was set up to evaluate the effects of different concentrations of ascorbic acid (AA) and citric acid (CA) (vitamin C) (0, 50, 100, 150, mg $l^{-1}$) on *C. avellana* L. hairy roots browning.

**Table 1. The different culture media used to improve the *C. avellana* hairy roots growth and development**

| Media | Basic Mineral Salt | Intensity | Vitamins | Intensity | Reference |
|---|---|---|---|---|---|
| WPM | Woody Plant Medium | Full | WPM | Full | Lloyd and McCown (1981) |
| $\frac{1}{2}$ WPM | Woody Plant Medium | $\frac{1}{2}$ | WPM | $\frac{1}{2}$ | Lloyd and McCown (1981) |
| $\frac{1}{4}$WPM | Woody Plant Medium | $\frac{1}{4}$ | WPM | $\frac{1}{4}$ | Lloyd and McCown (1981) |
| $\frac{1}{4}$WPM + vitB5 | Woody Plant Medium | $\frac{1}{4}$ | Gamborg's B5 medium | Full | Lloyd and McCown (1981), Gamborg *et al.* (1968) |
| SH | Schenk and Hildebrandt medium | Full | SH | Full | Schenk and Hildebrandt (1972) |
| $\frac{1}{2}$ SH | Schenk and Hildebrandt medium | $\frac{1}{2}$ | SH | $\frac{1}{2}$ | Schenk and Hildebrandt (1972) |
| $\frac{1}{4}$ SH | Schenk and Hildebrandt medium | $\frac{1}{4}$ | SH | $\frac{1}{4}$ | Schenk and Hildebrandt (1972), |
| $\frac{1}{4}$ SH + vit B5 | Schenk and Hildebrandt medium | $\frac{1}{4}$ | Gamborg's B5 medium | Full | Schenk and Hildebrandt (1972), Gamborg *et al.* (1968) |
| DKW | Driver Kuniyuki Walnut medium | Full | DKW | Full | Driver and Kuniyuki (1984) |
| $\frac{1}{2}$ DKW | Driver Kuniyuki Walnut medium | $\frac{1}{2}$ | DKW | $\frac{1}{2}$ | Driver and Kuniyuki (1984) |
| $\frac{1}{4}$ DKW | Driver Kuniyuki Walnut medium | $\frac{1}{4}$ | DKW | $\frac{1}{4}$ | Driver and Kuniyuki (1984) |
| NRM | NRM | Full | NRM | Full | Nas and Read (2004) |
| $\frac{1}{2}$ NRM | NRM | $\frac{1}{2}$ | NRM | $\frac{1}{2}$ | Nas and Read (2004) |
| $\frac{1}{4}$ NRM | NRM | $\frac{1}{4}$ | NRM | $\frac{1}{4}$ | Nas and Read (2004) |

***Measurement of hairy roots growth and tissue browning***

In this study, all experiments focused on promising C. *avellana* L. hairy roots growth, and two features (growth rate and tissue browning) were assessed. The hairy root growth rate was evaluated according to the following equation and tissue browning was calculated by using the non-parametric one-way Kruskal–Wallis test ($P < 0.05$). Browning tissue was represented on scales of 1-4 (yellow, light brown, dark brown, black). The data were analyzed by ANOVA using the SPSS (version 15.0) and Duncan's multiple range test ($P < 0.05$) was applied to the assessment of the statistically significant differences among the mean values. Excel software (2016 version) was used to drawing the graphs.

$$\text{Growth rate of dry weight (GRDW)} = \frac{\textbf{Secondary dry weight} - \textbf{Primitive dry weight}}{\textbf{Primitive dry weight}}$$

***Paclitaxel detection***

Ultimately, the presence of PC has been confirmed in hairy roots using high-performance liquid chromatography (HPLC). Paclitaxel content has been isolated according to the following steps: The freeze-dried hairy roots powders were ultrasonicated in 8 mL methanol for 30 min followed with the centrifugation (2500×g) to separate the debris. Then the supernatant was desiccated with a rotary evaporator and dissolved in 2 mL water and dichloromethane (1:1, v/ v), and centrifuged for 15 min at 2500×g. The isolated dichloromethane phase was evaporated under vacuum- and resolved in 100 μL HPLC grade methanol. It, finally, was filtered with a 0.45 μm syringe filter and injected into the HPLC system (Waters, Milford, MA 01757), with a C-18 column (Develosil, 250 × 4.5 [NW], Seto, Aichi, Japan) and UV detector at 227 nm). The solution of acetonitrile and water (60:40), at a flow rate of 1 mL $min^{-1}$ were set up to PC tracing. The genuine paclitaxel (Sigma-Aldrich, T7402) was used as an external standard.

**Results**

***Culture media amendment experiments***

Some media composition were assessed in regard of improving hairy roots growth in solid nursery media. The media composition significantly affected GRDW. Reducing salts in the culture medium on one hand and adding B5 vitamins to the culture medium on the other hand, improved the growth of the hairy roots. WPM basic medium had an enhancing effect on the hairy roots growth rate compared to SH, DKW, NRM media. $\frac{1}{4}$ strength of WPM significantly improved GRDW (49.8) (Fig. 1A). The lower growth rates was obtained in DKW (Fig. 1A). Vitamins of B5 medium have a significant effect on root growth. The alternation of the vitamins of the two superlative media, WPM and SH media, with B5 vitamins, highly augmented the growth of hairy roots. Accordingly, $\frac{1}{4}$ WPM + vit B5 resulted the in highest GRDW (63.1) (Fig. 2A).

The effect of nutritional elements of the culture media, including media main composition and intensities of salts and vitamin composition on hairy roots growth and development, were evaluated in the liquid proliferation medium, too. It can be interpreted from the results that the composition and intensity of the salts significantly affected growth indexes. The improved GRDW was obtained from SH basic media in comparison with NRM and DKW and WPM (Fig. 1B). The lowest GRDW (0.62) was obtained in DKW. Besides, reducing the salt intensity significantly affected the growth rate of hairy roots. Accordingly, the reduction of salt concentration to $\frac{1}{4}$ intensity in media improved the growth rate. The highest GRDW (49.8) were obtained from $\frac{1}{4}$ SH media (Fig. 1B). Similar to solid nursery media, the composition of vitamins in media has a significant impact on the growth rate of hairy roots in liquid media. The exchange of original vitamins composition of media to B5 medium vitamins improved the hairy roots growth rate in both SH and WPM media. Significantly, the best GRDW (63.14) have been obtained from $\frac{1}{4}$ SH + vit B5 (Fig. 2B).

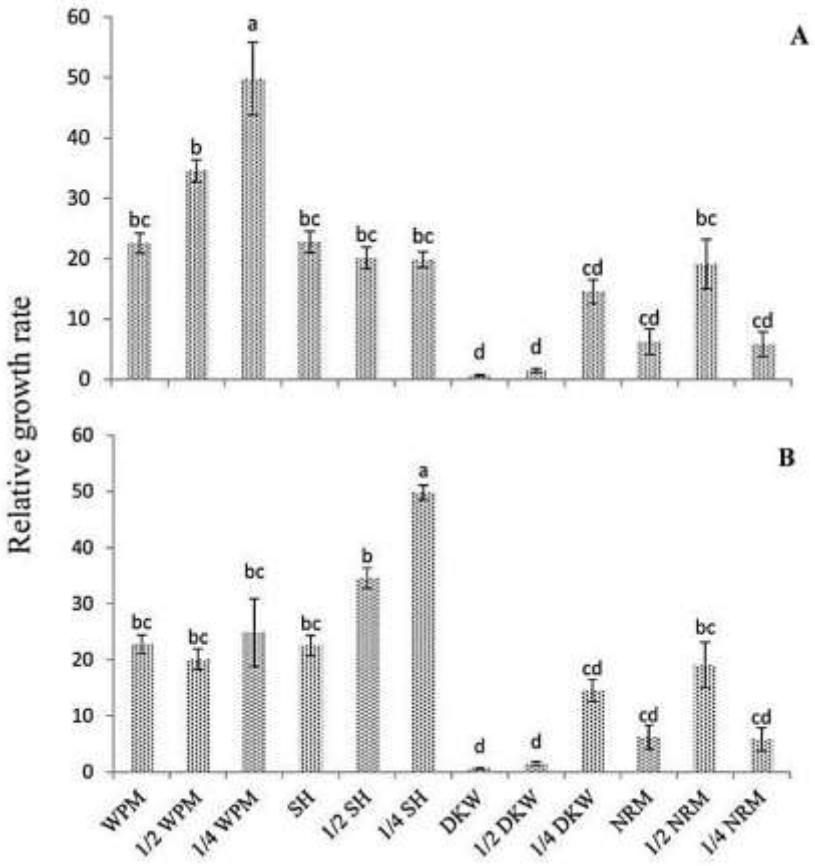


**Fig. 1.** Effect of different culture media on the growth rate of the dry weight of hazelnut hairy roots in (A) solid nursery medium, (B) liquid culture medium; Duncan's multiple range test was applied to the comparison of the means. Different letters on the bars refer to significant differences.

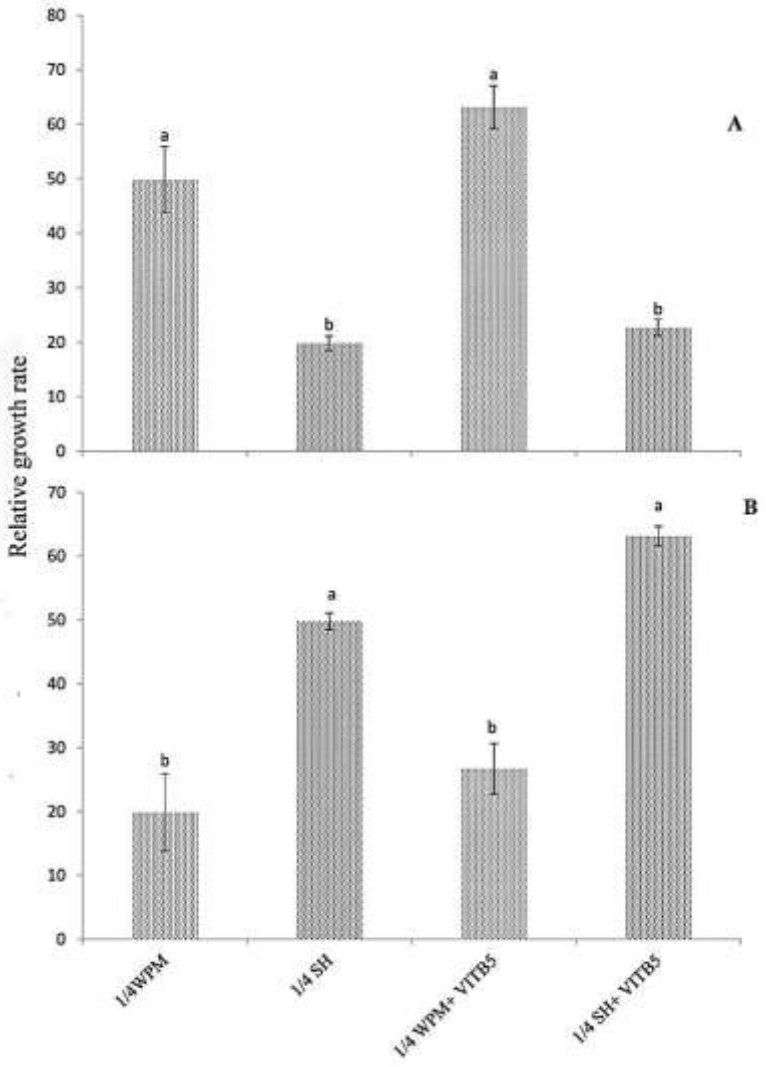


**Fig. 2.** Effect of replacement of SH and WPM media vitamins with B5 medium vitamins in (A) solid nursery media, (B) liquid culture medium on the growth of the hairy roots; Duncan's multiple range test was applied to compare the means. Different letters on the bars refer to significant differences.

Generally, during hairy roots induction, the browning of the explants started from the wound site and spread over the explants and hairy roots. Another experiment was performed to assess the effects of the different concentrations of ascorbic acid (AA) and citric acid (CA) (vitamin C) (0, 50, 100, 150, mg $l^{-1}$) on *C. avellana* L. hairy roots browning. Browning of the hairy roots occurred 2-3 days after hairy roots transferring in liquid media. A significant difference was found between the control (0 mg $l^{-1}$ AA + CA) and applying the combination of anti-browning agents, interpreted from the univariate analysis (Kruskal–Wallis test) (Chi-square: 14.3; Asymp. Sig: 0.003) which indicated that application of AA + CA decreased the amount of hairy roots browning and the lowest browning index was observed at 100 mg $l^{-1}$ AA + CA (Fig. 3). A non-significant reduction of hairy root browning was detected at a low concentration of AA + CA (50 mg $l^{-1}$) (Fig. 3). The high concentration of AA + CA (150 mg $l^{-1}$) weakened the growth rate (Fig. 4).

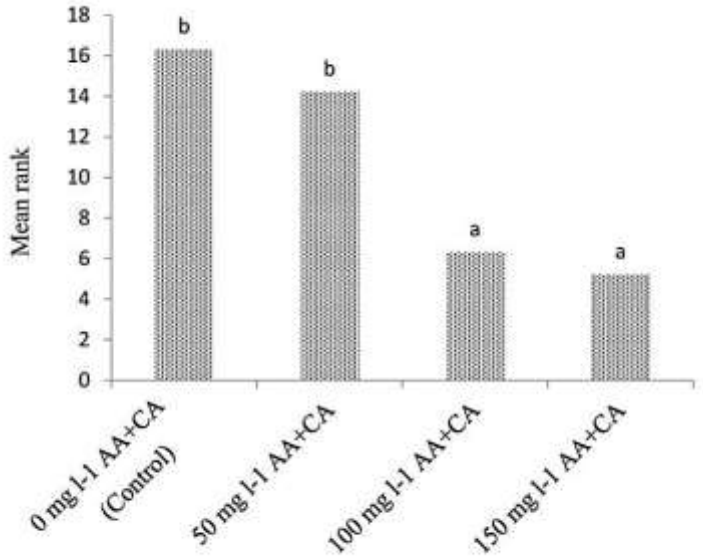


**Fig. 3.** Effect of antioxidants (AA + CA) application on hairy roots browning of hazelnut in liquid proliferation medium. The analysis was done with the Kruskal–Wallis test. Treatments with different letters are significantly different ($p < 0.05$). AA: ascorbic acid, CA: citric acid.

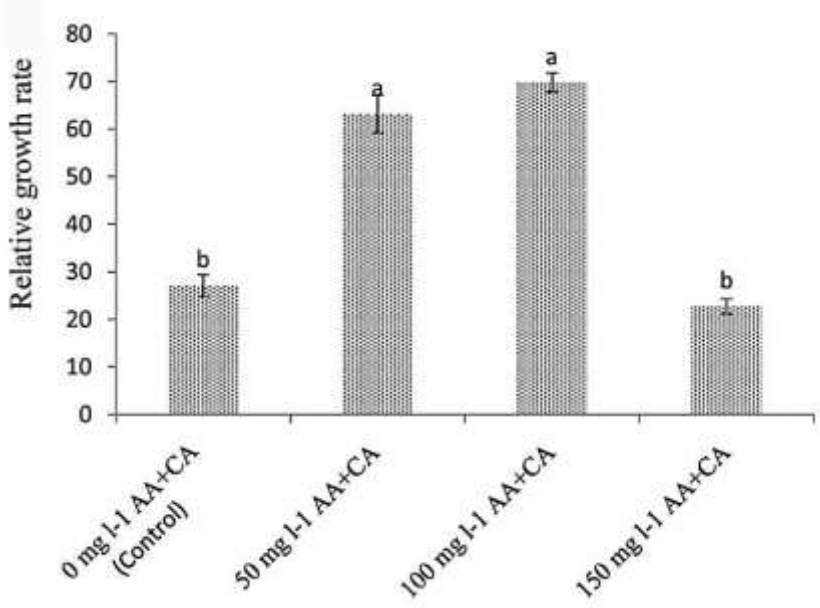


**Fig. 4.** Effect of antioxidants (AA + CA) application on the growth rate of hazelnut hairy root culture in liquid proliferation medium. Duncan's multiple range test was applied to compare the means. Different letters on the bars refer to significant differences ($p < 0.05$). AA: ascorbic acid, CA: citric acid.

***Paclitaxel detection***

HPLC analysis was used to confirm the intercellular PC content of hairy roots in the present study. The total yield of PC concentration in the non-elicited hairy root was evaluated 4.02 µg $g^{-1}$(DW) at the retention time ($t_R$) 8.3 min.

**Discussion**

The effect of culture media, different intensities of basal compounds, and combinations of vitamins in culture media on *C. avellana* hairy roots growth and development were studied. Culture media amendment studies were designed in three independent experiments. In the solid nursery culture media conditions, regarding the rate of secondary dry weight to primary dry weight (GRDW) of hairy roots, besides the culture media, using the lower concentration of nutrients significantly increased GRDW. Accordingly, ¼ WPM medium was the most effective, and DKW was the least efficient medium among all assesd media. As well, vitamin composition in culture media significantly affected the growth rate. The alternation of vitamin composition in culture media with Gamborg's B5 medium vitamins highly improved the growth rate of hair roots, and ¼ WPM + vitB5 was even more effective than ¼ WPM. In the section which the hairy roots transferred to liquid media to proceed with the growth and development, similar results were obtained. Reduction of the concentration of nutrient media had an influential impact on the growth rate with the difference that ¼ SH media was the best of all media. Additionally, ¼ SH + vitB5 improved the GRDW in comparison with ¼ SH. The effect of culture media and the intensity of nutrients for thriving culture vary for different purposes and plant species. Several efforts to improve hazelnut tissue culture have indicated DKW and NRM are efficient *C. avellana* multiplication. Shoot proliferation was superior on a modified DKW medium or a combination of DKW medium and WPM to MS, WPM, Perez-Tornero medium (PT) (Perez-Tornero *et al.*, 2000) and Anderson (1983) medium (Damiano *et al.*, 2005; Yu and Reed, 1993). Also, Nas and Read Medium (NRM), a newer medium formulated based on hazelnut kernel composition, was introduced as an efficient medium for hazelnut micropropagation (Nas and Read, 2004). The superior callus induction was obtained from NRM medium (Shirazi *et al.*, 2020). In contrast, the solitary study in hazelnut hairy roots indicated that WPM medium was significantly more effective in hairy roots induction than DKW and MS media (Jalalipour Parizi *et al.*, 2020). Our results, supportingly, showed that WPM media improved hairy roots growth rate in solid nursery culture media, and also DKW and NRM are inferior ones. SH media has been used to *Genista tinctoria* hairy root culture (Łuczkiewicz and Kokotkiewicz, 2005), and performed the best for *Angelica gigas* (Xu *et al.*, 2009), *Polygonum tinctorium* Lour (Young-Am *et al.*, 2000), and *Plumbago zeylanica* (Sivanesan and Jeong, 2009) hairy roots. Diluted SH media increased the growth rate of hairy roots in *Catharanthus roseus* (Jung *et al.*, 1994), *Scutellaria baicalensis* (Leea *et al.*, 2013), *Angelica gigas* (Xu *et al.*, 2009). Similar to the results of the present study, the diminution of the intensity of culture media nutrients performed the best growth rate in *C. avellana* hairy roots induction (Jalalipour Parizi *et al.*, 2020).

In general, during hairy roots induction, the browning of explants started from the wound site and spread over the explants and hairy roots. Exogenous AA and CA are included in antioxidants compounds that reduce oxidative stress and impede oxidation of phenolic compounds and, subsequently prevent tissue browning (Smirnoff, 2005). While, these compounds exert a negative effect on *Agrobacterium* growth in co-culture medium (Rana *et al.*, 2016), minimize the explants browning and hairy roots browning (Hassan

and Belbasi, 2017). Some studies revealed that application of the combination of AA and CA was remarkably efficient to inhibit the hairy roots browning (Hassan and Belbasi, 2017). It has been inferred from our results that, a combination of 100 mg $l^{-1}$ AA + AC reduces the hazelnut hairy roots browning and improves hairy roots growth. However, higher concentrations of AA + CA not only did not have significant effects on the hairy roots browning; but also reduced root growth.

**Conclusions**

The growth rate of hazelnut hairy roots has been improved by modification of culture media compositions. The culture media, the intensity of the mineral salts, and the combination of vitamins significantly affected the growth of hairy roots. Accordingly, ¼ WPM + vitB5 and ¼ SH + vitB5 were the efficient media for hairy roots growth and development in solid nursery and proliferation liquid culture media, respectively. Furthermore, the application of 100 mg $l^{-1}$ AA + CA reduced the browning of hairy roots and improved their growth rate.

**Conflict of interests**

The authors declare that they have no competing interest.

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