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Impact of different culture media on hairy roots growth of *Valeriana officinalis* L.

Ali PAKDIN PARIZI[1*], Mohammad FARSI[1], Ghorban-Ali NEMATZADEH[2], Amin MIRSHAMSI[1]

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ABSTRACT

Transformed hairy root cultures of *Valeriana officinalis* were established by infection with *Agrobacterium rhizogenes* strain ATCC 15834. To determine the effect of different media on the growth of *V. officinalis* hairy roots, MS, B5 media (1.0X and 0.5X strength), N6 medium and a modified MS medium without phytohormones were used. In addition, different NH_4^+ to NO_3^- ratios in MS medium were studied. The effects of these treatments were evaluated after 21 days of culture in relation to hairy root growth. B_5 and ½ B_5 media were the best basal media for hairy root growth. MS medium supplemented with a 20:20 ratio (mM) of NH_4^+ to NO_3^- displayed highest growth rates and biomass yield in hairy root cultures. The present study demonstrated that the composition of culture medium and the ratio of different nitrogen sources have significant impact on the growth of *V. officinalis* hairy roots.

Key words: *Valeriana officinalis,* hairy root, medium composition

IZVLEČEK

VPLIV RAZLIČNIH GOJITVENIH GOJIŠČ NA RAST TRANSFORMIRANIH KORENIN ZDRAVILNE ŠPAJKE (*Valeriana officinalis* L.)

Transformirana kultura korenin zdravilne špajke je bila vzpostavljena z bakterijsko okužbo *Agrobacterium rhizogenes*, sev ATCC 15834. Preučevana so bila različna gojišča MS, B5 (1,0X in 0,5X koncentracija), N6 in modificiran MS brez fitohormonov. Dodatno so bila v MS gojišču preučevana različna razmerja med NH_4^+ in NO_3^-. Učinki teh tretmajev na rast transformiranih korenin so bili ovrednoteni po 21 dneh. B_5 in ½ B_5 sta bili najboljši osnovni gojišči za rast transformiranih korenin. MS gojišče dopolnjeno z dušikovimi spojinami v razmerju 20:20 (mM) NH_4^+: NO_3^- je vplivalo na največjo rast in biomaso korenin. Raziskava je pokazala, da imata sestava rastnega gojišča in različna razmerja dušikovih spojin značilen vpliv na rast transformiranih korenin zdravilne špajke.

Ključne besede: *Valeriana officinalis,* transformirane korenine, sestava rastnega gojišča

1 INTRODUCTION

Valeriana officinalis L. (valerian) is a perennial herbaceous and rhizomatous medicinal plant native to Europe and Asia which has been naturalized in eastern North America and cultivated on a commercial scale in these regions (Cronquist, 1981). *V. officinalis* has been longley used as an important source of pharmaceutical compounds in traditional medicine (Straube, 1968; Morazzoni and Bombardelli, 1995; O'Hara et al., 1998) e.g., clinical trials have shown that valerian extract is effective in treatment of mild to moderate sleeping disorders and it can encourage sleep and improve sleep quality (Leathwood and Chauffard, 1985; Schulz et al., 1994). Furthermore, the valerian root is considered as mild anodyne, anticonvulsant, antispasmodic, carminative and hypotensive (Capasso and DeFeo, 1996; Hiller, 1996). Potential mechanisms for the pharmacological activity of

[1*] Ferdowsi University of Mashhad, Biotechnology Faculty, Agriculture Department, Mashhad, Iran, ali.pakdin@gmail.com

[2] Genetics and agricultural Institute of Tabarestan, Sari, Mazandaran, Iran

valerian extracts may include increased release of γ-aminobutyric acid (GABA) and agonistic activities on the GABA receptors (Marder et al., 2003).

Sesquiterpenes of the volatile oil (valerenic acid and its derivatives, valeranone, valeranal) and valepotriates (valtrate, didrovaltrate, acevaltrate, iso-valeroxyhydroxyvaltrate) are two main groups of compounds in the subterranean organs of valerian(Goppel, 2004). The valerenic acids have been reported for the sedative activity of *V. officinalis* and they are often used as an indicator of medicinal quality (Hendriks et al., 1981). The main representative of these compounds are valerenic acid (VA), acetoxyvalerenic acid and hydroxyvalerenic acid (Bos et al., 1996).

Genetically transformed hairy roots obtained by infection of plants with *Agrobacterium rhizogenes* are suitable source for production of bioactive molecules due to their genetic stability and generally show fast growth in culture media free of growth hormones (Shanks and Morgan, 1999). The hairy roots often exhibit about the same or greater biosynthetic capacity for secondary metabolite production as compared to their parent plants, hence hairy roots have been used as an alternative and attractive method for the production of several plant metabolites (Zhou et al., 2011). Several attempts have also been made to enhance hairy root growth and their production of important bioactive compounds (Yu et al., 1996; Yu et al., 2006; Satdive et al., 2007; Shinde et al., 2009). Optimizing the composition of inorganic nutrients of the media for hairy root cultures is essential to gain high production of secondary metabolites (Condori et al., 2010; Shinde et al., 2010). It has been reported that concentration of nitrogen and appropriate ratio of nitrogen sources in the culture medium greatly affected the growth and production of secondary metabolites in hairy root cultures (Oksman-Caldentey et al., 1994; Yu et al., 1996; Lourenco et al., 2002).

In the present study, the effect of different basal media and various NH_4^+ to NO_3^- ratios in the culture medium on the growth of hairy root cultures of *V. officinalis* are discussed.

2 MATERIALS AND METHODS

2.1 Hairy root induction

Cotyledons of in vitro grown sterile *V. officinalis* L. plants were cut into pieces of approximately 1 cm in length. The explants were immersed in a suspension of *A. rhizogenes* strain ATCC 15834 containing 100 µM acetosyringone for 10 min and then blotted on sterile filter papers. All explants were then placed in petri dishes containing 25 ml of half strength solidified MS medium for co-cultivation. After 48 hours incubation in the dark at 28 ˚C, explants were transferred to fresh solidified ½ MS medium containing 500 mg l^{-1} cefotaxime and were subcultured at two week intervals to eliminate the bacteria. Tips of hairy roots were exsected and transferred to 50 ml, liquid ½ MS medium and were incubated at 25 ˚C on a rotary shaker at 110 rpm in darkness. Among several hairy root lines established, line No. 9 was selected for its vigorous and sustained growth and used for further experiments.

2.2 PCR analysis of hairy roots

Genomic DNA was extracted from the hairy roots of line No. 9 and from the roots of a non-transformed plant, to serve as a negative control, using the DNeasy Plant Mini kit (Qiagen, USA). Two 20-mer oligonucleotide primers, 5́gctcttgcagtgctagattt3́ (forward) and 5́gaaggtgcaagctacctctc3́ (reverse), were used for PCR amplification of the *rolB* and *rolC* genes. In addition, primers 5́atgtcgcaaggcagtaagccca3́ (forward) and 5́ggagtctttcagcatggagcaa3́ (reverse), amplifying a fragment of *vir*D2 gene were used for detecting bacterial contamination in hairy roots. The PCR reactions were carried out in a total 25 µl volume and consisted of 100 ng of genomic DNA, 10 µM each primers, 0.2 mM dNTP mix, 1 unit of *Taq* DNA polymerase and 2 mM $MgCl_2$. PCR condition was as follows: 94 ˚C for 3 min (initial denaturation), 35 cycles of 94 ˚C for 45 s, 56 ˚C for 1 min and 72 ˚C for 1 min and a final extension at 72 ˚C for 7 min. The PCR products were

separated by electrophoresing on a 1.2 % agarose gel in TBE buffer.

2.3 Media composition

Standard Gamborg's (B5), Murashige and Skoog's (MS) basal media, half strength medium of these media and N6 medium were used in the experiments. To determine the effects of different NH_4^+ to NO_3^- ratios on hairy root growth, conventional MS medium was modified in such a way that total NO_3^- and NH_4^+ ions were supplied from KNO_3 and $(NH_4)_2SO_4$ salts, respectively. Various combinations of this modified medium with different NH_4^+ to NO_3^- ratios (0:20, 10:20, 20:20, 20:10, 20:0 and 20:40 mM) were prepared. For investigating the effect of substituting NH_4NO_3 with $(NH_4)_2SO_4$, the modified medium containing 20:40 ratio and its half strength were compared with conventional MS (half and full strength) in all experiments. The pH values of the media were adjusted to 5.8±0.2 prior to autoclaving and the concentration of sucrose was 30 g l^{-1} for all media.

2.4 Growth measurement

Two hundred miligrams of hairy roots were added to 50 ml of each media as primary inoculum. After 21 days of cultivation at 25 °C at 110 rpm in darkness, hairy root clones were harvested to determine the fresh and dry weights. All experiments were replicated three times and four erlenmeyer flasks were used for each treatment.

2.5 Statistical analysis

In all of the experiments, the layout was totally randomized. Analyses were performed using SAS V. 9 software package (SAS Institute Inc., Cary, NC, USA.). For comparing different treatments, a one-way analysis of variance (ANOVA) and Duncan test with a critical value of $P \leq 0.05$ were applied.

3 RESULTS AND DISCUSSION

3.1 Establishment of hairy root clones

Hairy roots induced by *A. rhizogenes* provide an alternative system for the production of valuable bioactive compounds because of their genetic and biochemical stability (Zhou et al., 2011). In the present study, hairy roots were successfully induced by infection of cotyledon segments of *V. officinalis* with *A. rhizogenes* strain ATCC15834. Hairy roots emerged from the infected sites within 12-15 days with 30% transformation frequency and maintained on hormone free ½ MS medium. Among the hairy root clones, clone No. 9 was selected on the basis of its growth rate. The selected transformed clone showed rapid growth rate and tendency for profuse branching and active elongation, whilst untransformed roots did not show similar growth, elongation or branching pattern (Figure 1).

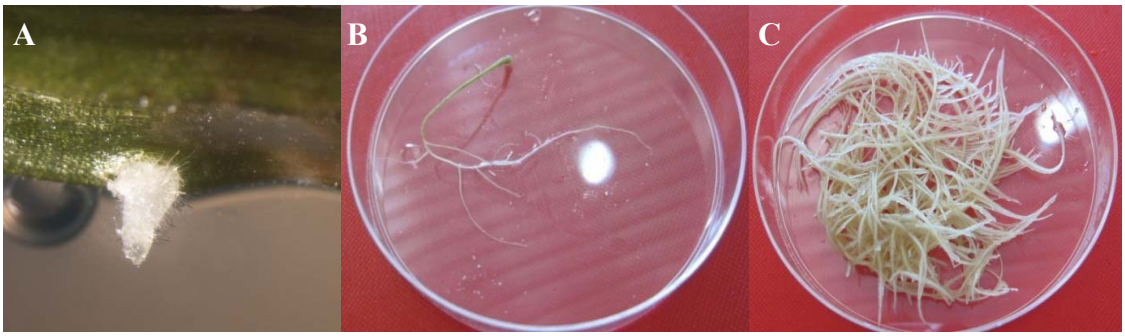

Figure 1: A) Hairy root formation on *V. officinalis* explant after 7 days of inoculation. B) non-transformed root after 4 weeks culture in ½ MS medium. C) Hairy roots after 4 weeks culture in ½ MS medium.

3.2 Molecular analysis

PCRs were performed with specific primers to determine presence of T-DNA segment of Ri plasmid in the genomic DNA of *V. officinalis* hairy roots. The PCR with primers specific for *rol*B and *rol*C genes and template DNA from hairy roots amplified the expected bands of 450 and 700 bp, respectively (Figure 2) confirming the successful integration of T-DNA, while DNA templates from untransformed roots (used as control) did not show any amplification. The PCR analysis of hairy root clones also revealed that no band was amplified for *vir*D2 gene (Figure 2c), indicating absence of *A. rhizogenes* ATCC 15834 contamination in the cultures.

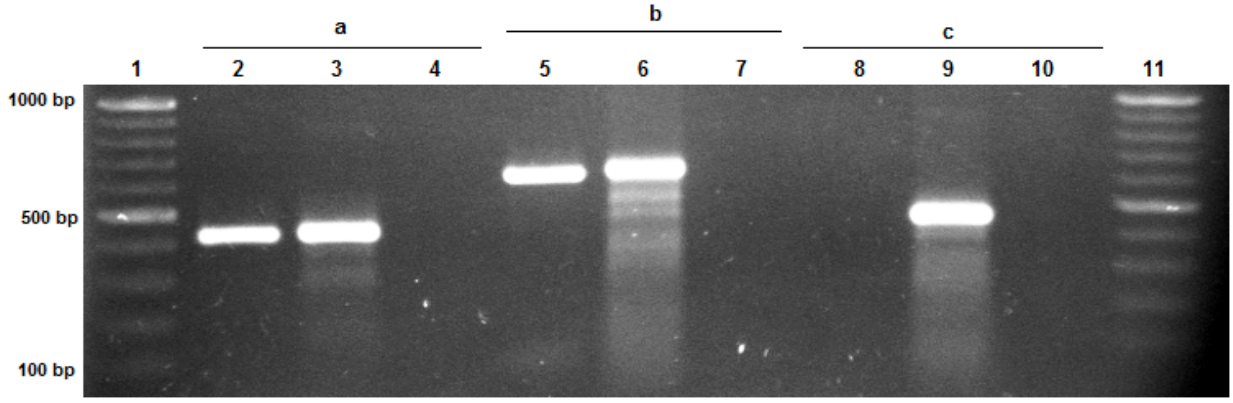

Figure 2: a) PCR amplification of *rol*B gene, lane 2: DNA from hairy roots; lane 3: *A. rhizogenes* DNA (positive control); lane 4: DNA from non-transformed root (negative control). b) PCR amplification of *rol*C gene, Lane 5: DNA from hairy root; lane 6: *A. rhizogenes* DNA; lane 7: DNA from non-transformed root. c) PCR amplification of *vir*D gene, Lane 8: DNA from hairy root; lane 9: *A. rhizogenes* DNA; lane 10: DNA from non-transformed root. Lanes 1and 11: Molecular size marker (100 bp ladder, Fermentas Co., Germany).

3.3 Effect of basal medium on hairy root growth

Based on previous studies, media composition could have a significant impact on hairy root growth in culture systems (Yu et al., 1996; Lourenco et al., 2002; Sivakumar et al., 2005). In the present study, the growth rate of hairy root cultures in different media was measured after three weeks. B5 and ½ B5 media produced the highest dry weight of hairy roots, 3.85 and 3.67 g l^{-1}, respectively (Figure 3). Although, biomass production in B5 medium was higher than ½ B5 medium, this difference was not statistically significant. The N6 medium was the weakest medium for root growth of *V. officinalis* with just 0.86 g l^{-1} dry weight after 21 days. Both ½ MS and ½ MSV media had similar effects on the growth of hairy roots and this result was also observed for full concentration of these two media. These results indicated that substituting NH_4NO_3 with $(NH_4)_2SO_4$ in MSV medium had no significant effect on root growth. Furthermore, increased K^+ ion concentration in the medium due to this replacement had not substantial alteration on the hairy root cultures. As shown in the figure 3, concentration of MS and MSV basal media influenced the biomass production. In both media higher dry weight values were obtained by roots grown in half strength MS and MSV media, 2.22 to 1.62 g l^{-1} and 2.658 to 2.01 g l^{-1}, respectively. These results are in contrast with the results of Russowski et al. (2006) that studied the growth of whole plant in liquid culture and Chen et al. (2003) for taxol production in cell cultures of *Taxus yunnanensis*. High concentrations of inorganic nutrients in the full strength MS and MSV media may be the cause of these results. However, hairy roots dry weight in ½ MS and MSV were not significantly different (Figure 3).

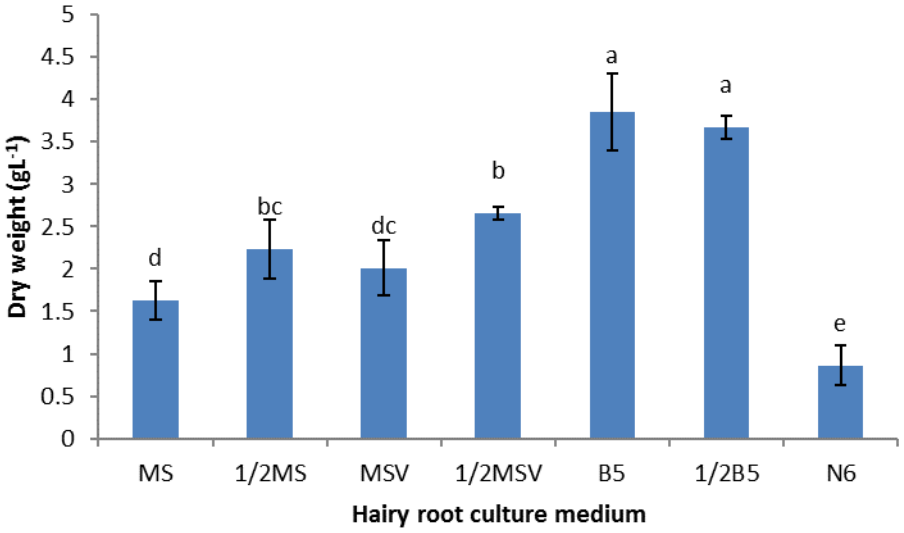

Figure 3: Effect of different culture media on hairy root growth of *V. officinalis*. Results are the mean of three replicates ± SD. Means with the same letter are not significantly different ($p > 0.05$).

3.4 Effect of NH_4^+ to NO_3^- ratio on hairy root growth

Different ratios of NH_4^+ to NO_3^- in MS medium were used for supplying the nitrogen requirement of the hairy roots. Figure 4 shows the effect of different nitrogen forms ratios on hairy root growth of *V. officinalis*. MS media supplemented with a 20:20 mM and 20:40 mM ratio of NH_4^+ to NO_3^- produced the maximum biomass after 21 days, 1.80 and 1.62 g l^{-1}, respectively. MS medium with a 20:20 ratio of NH_4^+ to NO_3^- slightly had greater influence on the growth of hairy roots than basal MS medium (20:40 mM), but this difference was not statistically significant. On the other hand, Shinde et al (2010) observed the highest biomass of *Psoralea corylifolia* hairy roots when MS medium was supplemented with NH_4^+ and NO_3^- at a ratio of 20:10. Decreasing the NH_4^+ concentration to 10 and 0 mM in the medium significantly reduced the hairy root growth. Similarly, decreasing the NO_3^- concentration had the same effect on the hairy root growth. Therefore, a balanced ratio of NH_4^+ to NO_3^- is an important factor for enhancing the growth of hairy root cultures in the *V. officinalis*.

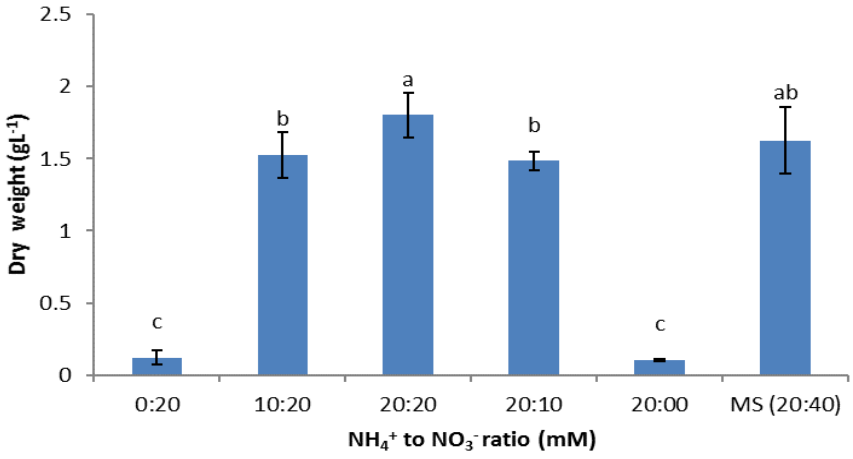

Figure 4: Effect of NH_4^+ and NO_3^- ratio on hairy root growth of *V. officinalis*. Results are the mean of three replicates ± SD. Means with the same letter are not significantly different ($p > 0.05$).

4 CONCLUSION

The present study highlights the importance of optimizing the culture media composition for *V. officinalis* hairy roots. The growth of valerian hairy roots was greatly affected by the composition and concentration of the culture media. The valerian hairy root system provides a promising platform that preserves the production of valuable sesquiterpenoids such as Valerenic acid.

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