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Agrobacterium transformation of *Rhodiola sp.*: current status and limitations

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Summary: The study of secondary metabolites has led to the discovery of new drugs for treating human diseases. However, consistent plant supply can be challenging, leading to the use of plant tissue culture techniques such as hairy root culture. Hairy roots have stable genetics, lateral branching, and can produce secondary metabolites, including alkaloids, flavonoids, and terpenoids. Research on hairy roots as a subject began in the late 19th century, and for the last four decades, hairy roots have been utilized for producing secondary metabolites and recombinant proteins. This article focuses on *Rhodiola* species - genus of perennial plants that belongs to the family *Crassulaceae* - and its potential as a source of secondary metabolites using hairy root culture techniques. *Rhodiola* sp. is widely distributed throughout the Arctic regions of the Northern Hemisphere, with several species having significant medicinal properties. The article discusses the possible use of hairy root cultures for the production of *Rhodiola* secondary metabolites, including salidroside and rosavins, which have demonstrated significant pharmacological activity in various studies. The use of elicitation and genetic engineering techniques to boost secondary metabolite production in *Rhodiola* hairy roots is also explored. Overall, the article highlights the potential of *Rhodiola* hairy root cultures as a valuable source of secondary metabolites with medicinal properties. However, despite some studies *Rhodiola* hairy root induction and culturing still remains highly unexplored.

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Key words: *Agrobacterium rhizogenes*, glycosides, hairy roots, roseroot, rosavin, salidroside

Introduction

Metabolites are the byproducts of an organism's metabolism, and they can serve various purposes, like providing energy, contributing to structural components, signaling, influencing enzyme activity, defending against threats, and interacting with other organisms. Primary metabolites are directly involved in essential processes such as growth, development and reproduction, while secondary metabolites often have significant ecological roles (Bartwal et al., 2013). Secondary metabolites are produced by microorganisms, plants and animals. The study of these secondary metabolites has led to the discovery of new drugs for treating human diseases. Continuous screening of natural resources has the potential to discover further valuable secondary metabolic compounds. Nowadays, pharmaceuticals are often produced using metabolites derived from plants. However, meeting the growing demand for pharmaceuticals can be difficult due to inconsistent plant supply. The growth cycle of plants is too long and the amount of important secondary metabolites are usually low. To address this issue, scientists have adopted several plant tissue culture techniques like precursor feeding, elicitation, hairy root cultures (HRC's) etc. HRC involves using *Agrobacterium rhizogenes* to transfer DNA from the root-inducing plasmid into the plant's genome, resulting in the expression of a hairy root phenotype (Chandra 2012). The emergence of the agriculturally important neoplastic disease manifested with hairy roots (HRs) is attributed to the gram-negative, symbiotic bacteria known as *Rhizobium rhizogenes* formerly *Agrobacterium rhizogenes* (Gutierrez-Valdes et al., 2020). It was first discovered in the beginning of the 20th century as an invasive pathogen, when American researchers isolated bacteria cultures from the hairy roots of an apple tree (Smith et al., 1911). In the late 1970s, the application of this bacteria has revolutionized the field of plant biotechnology (Doran, 2013). The interaction between the host plant and the specific DNA fragment (*T-DNA*) of the bacterial (root-inducing) *Ri* plasmid, produces the HR's (Chilton et al., 1982; Gutierrez-Valdes et al., 2020). The transfer of the *T-DNA* into the nucleus of a host plant has found a wide spectrum of applications in the production of plant-derived molecules, recombinant proteins used as medicine, molecular breeding, and last but not least in environmental protection through phytoremediation (Georgiev et al., 2012; Gutierrez-Valdes et al., 2020). For the last four decades since the initial publications, hairy roots have been utilized for producing secondary metabolites including alkaloids, anthocyanins, flavonoids, ginsenosides, stilbenes, lignans, terpenoids, and shikonin, as well as recombinant proteins such as vaccines, monoclonal antibodies, and therapeutic proteins (Massa et al., 2019; Skarjinskaia et al., 2013; Donini et al., 2018; Zhang et al., 2019). Hairy roots are predominantly used as a source of pharmacologically significant secondary metabolites, often equal to or surpassing their content in intact plant roots. The high synthesis of diverse secondary metabolites is attributed to the presence of *rol*-genes, particularly *rol B* and *rol C*. When root cultures fail to synthesize enough secondary metabolites, elicitation or genetic engineering techniques are used to boost their production (Bhaskar et al., 2022)

The molecular background underlying the *R. rhizogenes* transformation involves several steps. First, an allelopathic communication through phenolic compounds generated from the roots of the host plant, attracts the bacterial cells to attach to the root cells. Next, a T complex from the *T-DNA* (T strands and proteins connected to them) is formed. Afterwards, the bacteria transfers the *T-DNA* via the T complex to the plant host genome, so that the *T-DNA* is integrated and expressed by the host's genome. Finally, the HRs develop within few days (Georgiev et al., 2012). At the molecular level, the transformation is quite complex with various genes taking place in the process. The *vir* region of *pRi T-DNA* and chromosomal virulent (*chv*) genes encompass the *Vir D1* and *Vir D2* coding for proteins that bind to and nick the DNA (Georgiev et al., 2012). Furthermore, other proteins encoded by *vir E1* and *vir E2* genes play a role as protectors of the *T-DNA* against nucleases from the host plant. Alternatively, other strains of the *R. rhizogenes* contain *pRi GALLS* gene which produces nuclear localization signal and helicase activity, instead of the missing *vir E1* and *vir E2* genes (Gelvin, 2009; Hodges et al., 2014). During the transfer and integration, the *T-DNA* contains two sequences named left (*TL*) and right (*TR*) border (Chandra, 2012). However, only the presence of the *TL* sequence induces HRs (Georgiev et al., 2012). Four open reading frames on the *TL* sequence are responsible for the transformation and HR induction, namely *rol A, B, C,* and *D* genes (Georgiev et al., 2010). The *root locus* genes (*rol*) are virulent factors that induce hairy root expression and specifically affect root morphology. Each *rol* gene is specific to a particular phenotype and together they synergistically induce hairy roots to produce stronger effects compared to when expressed individually. The *rol A* gene encodes 100 amino acid proteins and causes wrinkled leaves, stunted growth, and extremely shortened internodes and rounded leaves (Roychowdhury et al., 2013). The *rol B* gene, which encodes a 316 amino acid protein, increases the sensitivity of the cells to auxin and stimulates flower and root formations (Bonhomme et al. 2000; Mano et al. 1989). The *rol C* gene, which encodes a 180 amino acid protein, results in dwarfed bushy plants with reduced apical dominance and increases cytokinin activity (Kodahl et al., 2016). The *rol D* gene, similarly to *rol B*, is a late-auxin induced gene. It encodes a 344-amino acid protein and has an ORF (open reading frame) of 1032 base pairs. However, when exposed to higher levels of auxin, the promoter induction of *rol D* is comparatively lower than that of *rol B*. The HR production involves cultivation of wounded explant, inoculated or co-cultivated with *R. rhizogenes* suspension. The detection of the *rol* and *Vir G* genes can be performed by PCR. In addition, the successful transformation after *R. rhizogenes* infection can be confirmed by southern blot hybridization and/or northern blotting (Georgiev et al., 2007; Georgiev et al., 2012). Hairy roots have stable genetics, lateral branching, and can grow well without hormones. It typically takes 5-7 days to see symptoms of hairy roots after infection, but the timing may vary depending on the type of explant and plant species used (Kifle et al., 1999; Kamada et al., 1986).

Hairy root cultures in *Rhodiola* L.

Rhodiola is a genus of perennial plants that belongs to the family *Crassulaceae*, with over 200 species in the genus, distributed throughout the Arctic regions of Europe, Asia, and North America. Around 20 of these species, including the most famous *Rhodiola rosea* ***(Figure 1.)*** along with further members of the genus like *R. alterna, R. brevipetiolata, R. crenulata, R. kirilowi, R. quadrifida, R. sachalinensis, R. sacra, R. algida* are commonly used as traditional medicine in Asia (Bawa et al., 2009). *Rhodiola* plant extracts have traditionally been utilized as a tonic, anti-inflammatory, antidepressant and adaptogen, which means it helps the body to adapt to stress by regulating the body's physiological response to stressors (Kelly 2001; Huanyue et al., 2021). In recent years, there has been growing interest in the therapeutic potential of *Rhodiola* species, particularly their ability to alleviate stress and improve cognitive function (Darbinyan et al., 2000). Various pharmacological effects, including neuroprotective, anti-inflammatory, and anti-tumor activities have been reported. For example, studies have shown that *R. rosea* ie. roseroot or golden root supplementation can improve cognitive performance in stressful situations, increase mental clarity and focus, and reduce mental fatigue. These findings have led to the investigation of *R. rosea* as a potential treatment for neurological disorders, such as depression, anxiety (Anghelecu et al., 2018), and Alzheimer's disease (Nabavi et al., 2016). *Rhodiola* species have been extensively studied for their potential health benefits. *Roseroot* based supplements have been commonly employed to enhance the stress resistance of astronauts. The roots and rhizome of the plant contain salidroside, tyrosol (which serves as its precursor), and cinnamic glycosides like rosin, rosavin, and rosarin. Additionally, flavonoids, tannins, and gallic acid, along with its esters, are significant components found in roseroot (Brown et al., 2002). Tyrosol and cinnamyl alcohol glycosides as the primary bioactive ingredients, have been reported to exhibit excellent biological activities. These activities include anti-stress activity against oxidative and endoplasmic reticulum stress (Li et al., 2012; Tao et al., 2016), anti-depression activity, anti-cancer activities towards lung, gastric, bladder, ovarian, and breast cancer (Ren et al., 2019; Yang et al., 2019a; Yang et al., 2019b; Yu et al., 2018), neuroprotective activities against Parkinson's, and Huntington's disease, cardio-protective and hepatoprotective activity (Wu et al., 2009; Li et al., 2022). Additionally, salidroside has been reported to have anti-inflammatory, anti-tumor, anti-aging and anti-viral properties. These studies suggest that salidroside could have the potential as a therapeutic agent for various diseases. The use of roseroot-based products has gained widespread commercial interest due to their documented pharmacological effects and safe use. However, meeting the growing industrial demand for these products has become a major challenge. Most of the raw materials derives from natural populations, mainly located in the Altai area of South Siberia, which are now severely threatened due to intensive collection (Marchev et al., 2016). Roseroot has been listed on the Red List of Russia and several other countries and collection is now highly regulated. Although collection in European countries is less economically significant, costs associated with collection and transportation are high because the natural habitat of this plant is in mountainous areas. Thus, the cultivation of roseroot appears to be the only viable solution to produce sufficient quantities of raw material for industrial purposes.

The only solution to meet the industrial demand for raw roseroot material appears to be through its cultivation. Cultivation trials have been conducted in various regions of the former Soviet Union, including Russia (Elsakov & Gorelova 1999), as well as in Sweden, Poland (Furmanowa et al. 1999), Finland, Canada and Germany. Even though currently the primary source of raw material is gathered from natural

populations, the question is if cultivation could provide enough raw material for industrial purposes in the future. Nonetheless, there are difficulties associated with the field cultivation of *Rhodiola*, such as the high costs of establishing fields through seedling transplantation, the long five-year cultivation period from planting to harvesting, and the labor-intensive harvesting and post-harvest processing of the root yield. Secondary metabolites that are important for commercial use are often found in plant roots, but they have complex structures and are present in small amounts, making them difficult to extract. Harvesting roots directly can be costly and also threatens the survival of the plant species. Therefore, an alternative method that can meet commercial demands while maintaining natural plant germplasm is needed. While biotechnological interventions like cell suspension cultures have been developed for secondary metabolite production, they have limitations such as metabolite production only in specific cell types and genetic instability. In contrast, hairy root cultures can produce a range of bioactive molecules and closely mimic intact root systems' production potential. HR cultures have other desirable properties like high genetic and biochemical stability, short doubling time, and the ability to produce novel compounds, making them a useful tool for various applications.

Figure 1. *Rhodiola rosea* in its natural environment.

Rhodiola is considered recalcitrant to gene transformation, but still, there are few reports (***Table 1.)*** on the Agrobacterium transformation of some of its species (Marítnez et al., 2020). However, in general, the transformation of *Rhodiola* is still underdeveloped. Successful production and characterization of hairy roots from *R. quadrifida* using *AR A4* strain was reported by Stepanova and colleagues (2021). They studied the growth characteristics and ability to biosynthesize the main phenolic metabolites (salidroside and rosavin). They compared the hairy roots and callus cultures initiated from them. Results showed that the hairy roots had twice the growth index of the callus culture. The salidroside content in the callus culture was comparable to its level in *R. quadrifida* rhizomes grown in natural conditions, indicating its potential for biotechnological use (Stepanova et al., 2021). *R. crenulata* was successfully transformed using the *AR C58C1*, and hairy root cultures were established (Lan et al 2013). Zhou et al. (2010) reported the successful establishment of hairy root culture from *R. sachalinensis*. The study found that hairy roots could be generated from cotyledons and cotyledonary nodes infected with *Agrobacterium Ri-plasmid* strains *A4*, *R1601*, and *ATCC 15834*. The hairy roots grew well in 1/2 MS liquid medium, scattered light, 20 °C, and pH 4.5-4.8. The study also found that the content of salidroside increased when grown in lower nitrogen substances, such as a 1:1 ratio of NH_4^+ to NO_3^-. It is reported that adding a precise concentration of precursor (such as tyrosol, tyrosine, and phenylalanine) and elicitor (such as *Aspergillus niger*, *Coriolus versicolor*, and *Ganoderma lucidum*) to the medium was necessary in order to achieve high salidroside content in *R. sachalinensis* hairy root cultures. By using an elicitor in the liquid MS medium and supplementing the precursor via chemical feeding, they found that biomass accumulation and salidroside production improved. The optimal concentration for the elicitor and precursor in the liquid medium was determined to be 0.05 mg/l and 1 mmol/l, respectively (Zhou et al., 2007). Yu and colleagues (2011) reported the impact of upregulating three *UDP-glycosyltransferases* genes (*UGT72B14*, *UGT74R1*, and *UGT73B6*) for salidroside biosynthesis in transgenic hairy root lines of *R. sachalinensis*. They found that *UGT72B14* played a significant role in salidroside production, with the highest activity levels detected in roots both *in vitro* and *in vivo*. *UGT73B6* also showed potential as a contributor to salidroside synthesis, with higher activity levels than *UGT74R1* and being highly expressed in roots. Moreover, the hairy root culture system demonstrated greater salidroside production levels compared to the transgenic calli and transgenic plants expressing *UGT73B6*. One study reported the transformation of *R. rosea* and two accessions of *R. pachyclados* with *A. rhizogenes* to develop putatively transformed roots. The growth of putatively transformed roots in simulated bioreactors including various concentrations of IAA was used to improve their growth. Of the inoculated stem and leaf explants, *R. pachyclados* accession - developed the highest percentage of roots, and *R. rosea* had a lower percentage but with significant differences in root development between stem and leaf explants. The putatively transformed roots increased in weight and were indistinguishable from the control roots (Himmelboe et al., 2015). Induction of hairy root cultures in *R. kirilowii* by *Agrobacterium* transformation of wounded seedlings was reported by Grech-Baran et al. (2014) The report suggests that the hairy roots are more potent and abundant source of rosavin in comparison to non - transformed roots. However, none of the examined phenolic glycosides were formed in roots or secreted to the post-culture media without exogenous cinnamyl alcohol supplementation. The authors reported that the addition of cinnamyl alcohol enabled the production of rosarin, rosavin, and rosin in non-transformed wild-type root cultures and rosarin and rosin in hairy cultures. The study also found that feeding cultures with cinnamyl alcohol on the inoculation day and additional sucrose on day 14 of the growth cycle resulted in increased rosavin production. For rosin accumulation, the most advantageous strategy was adding cinnamyl alcohol alone on 14th day. Nonetheless, there are reports on the successful transformation of *R. rosea* with *Rhizobium rhizogenes* Agropine strain *ARCC43057* inducing hairy roots. The study also evaluated the effect of varying geographical locations, genotypes, and morphologies on the induction of hairy roots in *R. rosea*. The study showed that light had a beneficial impact on the survival of *R. rosea* leaf explants following bacterial inoculation, leading to higher rates of hairy root formation. Furthermore, the response to inoculation varied among *R. rosea* plants from different geographical locations, genotypes, and morphologies (Marítnez et al., 2020). Despite the induction of hairy roots in *R. rosea*, there is not any study to date, that reports the successful establishment of hairy root cultures in *R. rosea*.

Table 1. Summary of the available literature aiming hairy root formation in *Rhodiola* species.

Species	Experiment	Reference
R. sachalinensis	Establishment of hairy root culture with *A4, R1601,* and *ATCC 15834* strains treatment with precursors and elicitors	Zhou et al. (2010)
R. sachalinensis	Upregulation of three salidroside biosynthesis related genes	Yu et al. (2011)
R. crenulata	Hairy root cultures were established with *AR C58C1* strain	Lan et al. (2013)
R. kirilowii	Hairy roots produced more rosavins upon precoursor treatment	Grech-Baran et al. (2014)
R. rosea and *R. pachyclados*	Putatively transformed roots	Himmelboe et al. (2015)
R. rosea	Transformation with Agropine strain *ARCC43057*	Marítnez et al. (2020)
R. quadrifida	Produced hairy roots with *AR A4* strain and compared salidroside content with callus cultures	Stepanova et al. (2021)

Conclusions

In conclusion, the demand for *Rhodiola* raw material especially *R. rosea* is increasing, for now it can only be met through its cultivation and collection from wild natural populations. Even though today gathering is the primary source of *Rhodiola rosea* raw material, it is not sustainable in the long run. The field cultivation of *R. rosea* is challenging and labor-intensive. An alternative method that can meet commercial demands while maintaining natural plant germplasm is needed. Hairy root cultures can produce a range of bioactive molecules and closely mimic intact root systems' production potential, therefore making them useful for various applications. *Rhodiola* is considered recalcitrant to gene transformation, but there are few reports on the *Agrobacterium* transformation of some of its species. Successful hairy root cultures have been established from *R. quadrifida, R. crenulata and R. sachalinensis* and different studies have shown that the salidroside content can be increased by manipulating the culture conditions or by upregulating specific genes related to salidroside biosynthesis. The successful establishment of HRCs from *Rhodiola* species especially *Rhodiola rosea* offers an opportunity for the sustainable production of its valuable bioactive compounds.

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