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ORIGINAL ARTICLE

Linalool as a novel natural factor enhancing ginsenoside production in hairy root cultures of American ginseng

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Abstract

Ginsenosides are triterpenoid saponins, accumulated in root of *Panax qiunquefolius*. These secondary metabolites have numerous pharmacological properties such as: antimicrobial, antioxidant, anti-inflammation, anticancer. They have been found to regulate the functioning of the nervous and endocrine systems, thus maintaining homeostasis. Root harvesting for ginsenoside extraction for pharmaceutical industry destroys the entire plant, limiting its natural occurrence and impacts on wild populations of ginseng. The present study showed that hairy root cultures of *P. quinquefolius*, after using linalool as elicitor, can increase ginsenoside yield without the use of field-grown plants and independently of the vegetative season. The content of seven ginsenosides (Rb1, Rb2, Rb3, Rc, Rd, Rg1, Re) was determined. We found linalool to stimulate most studied saponin accumulation regardless of exposure time (24 and 72 h). Shorter time of elicitation and 0.1 µM linalool in medium proved to be optimum conditions to obtain the highest total saponin content (29% higher level than that of untreated roots) and Rg-group metabolites (2.28 fold higher amount than untreated roots). Ginsenosides, belonging to protopanaxadiol derivatives, were found to have different dynamics of their content changes depending on linalool concentration. The highest increase in untreated roots was noted for compound Rd. Therefore, elicitation with linalool can be an effective method of enhancing ginsenoside production in *P. quinquefolium* hairy root cultures cultivated in shake flasks.

Key message

Elicitation with linalool can be an effective method of improving ginseng saponin production in *P. quinquefolium* hairy root cultures cultivated in shake flasks.

Keywords American ginseng · Hairy root cultures · Linalool · Elicitation

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Introduction

Ginsenosides—triterpene saponins are secondary metabolites that are found almost exclusively in the plant genus *Panax*. A long history of the use of species such as *Panax ginseng* or *P. quinquefolius* in traditional medicine has led to numerous investigations of pharmacological effects of ginseng compounds, conducted both in vitro and in vivo. These studies confirmed high therapeutic potential of ginsenosides proving their regulating action on the nervous, endocrine, cardiac and immune systems. Additionally, many reports indicated multiple properties of ginseng saponins, including antimicrobial, antioxidant, anti-inflammation, anticancer, radioprotective, and anti-aging (Liu et al. 2020). Most ginsenosides are classified as members of the dammarane family. Each ginsenoside has at least two (carbon-3 and -20) or three (carbon-3, -6, and -20) hydroxyl groups

free or bound to monomeric, dimeric, or trimeric sugars. Ginsenosides also exist as stereoisomers, depending on the position of the hydroxyl group on carbon-20. These metabolites are divided into two groups based on their chemical structures: protopanaxadiol (PD) and protopanaxatriol (PT). Sugar moieties in the PD group attach to the 3-position of the dammarane-type triterpine, including Rb1, Rb2, Rc, Rd, Rb3, Rh2, and Rh3; sugar moieties in the PT group attach to the 6-position of the dammarane-type triterpine, including Re, Rf, Rg1, Rg2, and Rh1 (Szczuka et al. 2019).

Initially, raw material for the production of medicinal ginseng products was obtained from natural places, but due to depletion of these resources, different attempts were made to cultivate ginseng; first, under natural conditions, and then, under field conditions (Liu et al. 2021). Due to a high demand and high prices (from 20 to 1105 dollars per kilogram) of ginseng root, as well as the fact that this raw material cannot be currently obtained from natural places (since 1975 ginseng has been included in the "Red Book of Endangered Species" in Russia and it has been protected by Convention on International Trade of Endangered Species of Wild Fauna and Flora CITES), surface area for field cultivation of ginseng has increased (Wills and Stuart 2001; Zhuravlev et al. 2010; Olson 2014; Liu et al. 2021; Chen et al. 2022). Soil cultivation of this plant is very labor-intensive. The process of obtaining valuable raw material takes a lot of time (minimum 3–4 years) and entails high costs associated with agrotechny and use of prophylactic plant protection treatments (ginseng is a plant extremely susceptible to fungal diseases, as well as eagerly attacked by pests) (Jia et al. 2009; Proctor et al. 2014). In vitro cultures provide well-standardized conditions which enable to faster obtain raw material that is rich in biologically active ingredients. In recent years, cultivation of hairy root culture has become a focus of interest. Numerous literature reports demonstrated that this type of roots can become an alternative way to obtain valuable secondary metabolites for field crops or cultures of cell suspensions. (Gutierrez-Valdes et al. 2020; Hussain et al. 2022) Hairy root cultures are characterized with advantages that cell cultures do not have (Hussain et al. 2022). They are characterized with rapid growth which does not need to be enhanced by additional phytohormones. This allows to produce a large amount of biomass in a relatively short time. Besides hairy root cultures are genetically stable and no drastic decline in metabolite accumulation was observed as the root line grows. Their valuable advantage, in comparison to suspension cultures, is tissue and structural differentiation which plays an important role in the normal course of metabolic processes, considering the fact that some metabolites are synthesized only in specialized organs of plants. Apart from optimal concentration of sugars, nitrogen or phosphorus in the medium, technological methods are important factors affecting the production of biomass and secondary metabolites (Gutierrez-Valdes et al. 2020). The elicitation process is one of most common technological methods. It consists in subjecting in vitro culture to activity of the elicitor. Elicitors in plant biology are extrinsic or foreign molecules often associated with plant pests, diseases, or synergistic organisms. Elicitor molecules can attach to special receptor proteins on plant cellular membranes. These receptors can recognize the molecular pattern of elicitors and trigger intracellular defense signaling. This response results in enhanced synthesis of metabolites, reduced damage, and increased pest resistance, disease, or environmental stress. Effectiveness of the elicitation process depends primarily on the interaction between the plant cell and the elicitor (Ramirez-Estrada et al. 2016). Besides, literature data indicate that parameters such as a type and concentration of the elicitor, duration of elicitation, age and line of the culture, cell lines, addition of growth regulators, nutrient composition and culture conditions affect performance of the elicitor. Action of elicitors is not specific; thus, their type and optimal condition for their acting have to be selected experimentally for each particular culture (Halder et al. 2019).

Linalool (other names: β-linalool, linalyl alcohol, linaloyl oxide, allo-ocimenol, and 3,7-dimethyl-1,6-octadien-3-ol) is a monoterpene compound of essential oil obtained from plants belonging to families, such as Lamiaceae, Lauraceae or Rutaceae . Being a safe compound linalool and its medical potential are extensively investigated. Linalool is applied in aromatherapy and various industries: food, pharmaceutical or cometic (Kamatou and Viljoen 2008).

Bearing in mind various biological properties of linalool, our team used this compound to improve ginsenoside production in hairy root cultures. We applied transformed roots of five-leaf ginseng (*P. quinquefolium*, American ginseng) as a model culture. The hairy root of *P. quinquefolium* may be an excellent source of ginsenosides as their content level is similar to the one observed in ginseng roots cultivated in the field. Our research focuses on American ginseng cultures of hairy roots primarily due to ginseng saponins and their therapeutic properties. Considering time and cost of obtaining these biologically active compounds, our breeding which uses derived cultures in vitro enables to obtain ginseng biomass in a much shorter period of time (only 28 days). Its production does not require agrotechnical work in contrast to production of valuable material derived from traditional cultivation (minimum three years) (Kochan et al. 2016, 2018a, b). In previous reports, we described an influence of some elicitors required to increase ginsenoside production in hairy roots of American ginseng (Kochan et al. 2017, 2018a, b). They include yeast extract (YE), methyl jasmonate (MeJa), abscisic acid (ABA) (Alcalde et al. 2022; Markowski et al. 2022), used to improve secondary metabolite contents in many in vitro cultures, and essential oil

compound - trans-anethole (*t*-A), which successfully intensified ginseg saponin accumulation for the first time (Kochan et al. 2018b). Obtained results encouraged us to search for other essential oil ingredients to get even higher-efficiency saponin synthesis in the studied cultures. The primary objective of the present study was to determine whether linalool can be a potential new elicitor in enhancing triterpene saponin production in *P. quinquefolium* hairy roots cultivated in shake flasks. This research estimated the optimum dose and elicitation time of linalool for effective ginsenoside biosynthesis in the studied cultures. I The role of linalool in accumulation of secondary metabolites has not been documented in any plant of in vitro cultures.

Materials and methods

Hairy root culture

Hairy root cultures of *Panax quinquefolium* were cultivated in 80 mL of B-5 medium (Gamborg et al. 1968) without hormone, modified according to Kochan et al. (2016) description (concentration of ions: $NH_4^+$and NO_3^- was reduced to half of their level in relation to the standard B-5 medium; PO_4^{3-} level was reduced by 1/4 in relation to its amount in the standard medium) The cultivation was carried out at 26 °C ± 2 °C in darkness on rotary shakers (100 rpm). The mean inoculum size was about 300 mg fresh weight (f.w.) and 28.0 mg dry weight (d.w.).

Elicitation process

The elicitation lasted 24 and 72 h. Different concentrations of linalool (Sigma-Aldrich) were added on 25 day of culture when the culture of hairy root of *P. quinquefolium* was in the stationary growth phase. Twelve linalool stock solutions were prepared. The elicitors were diluted with 96% ethanol and then filter-sterilized using a sterile syringe filter Millex GS 0.22 µm (Millipore). The same volume of each linalool stock solution was added to 80 ml of medium to obtain the final linalool concentration: 0.01, 0.1, 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000 µM. Non-elicited cultures were regarded as control and ethanol was added to them. Each treatment was performed in three flasks and the experiment was repeated three times.

Ginsenoside content determination

Sample preparation

After the elicitation process, hairy roots were harvested, rinsed with distilled water, dried at room temperature. The plant material prepared in this way was used for ginsenoside extraction. To obtain crude methanolic extracts of ginsenosides, 1 g of hairy roots was extracted three times with 50 ml of 80% methanol for 30 min at solvent boiling temperature under a reflux condenser. Next, the extracts were evaporated to dryness in a vacuum evaporator and purified using an SPE column with octadecyl (C18) as a reverse phase. The dried methanolic extracts were dissolved in 50% HPLC methanol and placed on the column. The impurities were removed by rinsing with 30% HPLC methanol and H_2O. Ginsenosides were selectively eluted using 10 ml 100% HPLC methanol and subject to a quantitative analysis (Kochan et al. 2017).

Quantitative analysis of ginsenosides using HPLC method

Quantitative analysis of seven ginsenosides: Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1 (all purchased from Aldrich Sigma, Germany) was described in detail in a previous paper (Kochan et al. 2018a). It was carried on Agilent Technology 1200 liquid chromatography apparatus consisting of a LiChroART ® 250-4, Waters 600 Controller pump and UV-VIS Waters 996 detector combined with a Pentium 60 PC running Windows Millenium software. Ginsenosides were separated on a reverse C18 column. A mobile phase, composed of acetonitrile (A) (J.T. Baker, Deventer, the Netherlands) and water (B) (J.T. Baker, Deventer, the Netherlands), was used in gradient elution program: 0–16 min: 18% A, 82% B; 17–28 min: 30% A, 70% B; 29–60 min: 32% A, 68% B; 61–64 min: 80% A, 20% B; 65–68 min: 18% A, 82% A. The flow rate was 2 mL min^{-1}. The quantitative content of ginsenosides (mg g^{-1} d.w.) was determined by comparing retention time and peak areas between standards and samples.

Statistical analysis

All treatments were performed in triplicate. Data were analyzed using the Kruskal-Wallis test. Any relationships were considered significant for $p \leq 0.05$. Statistica Version 13.1 software was used for all statistical analyses (STATSoft, Tulsa, OK, USA).

Results

The present study reveals that linalool can increase ginsenoside production in *P. quinquefolium* hairy roots cultivated in shake flasks. The content of Rb1, Rb2, Rb3, Rc, Rd (protopnaxadiol derivatives, Rb-group ginsenosides), and Rg1, Re (protopanaxatriol derivatives, Rg-group ginsenosides) was determined after 24 h and 72 h elicitation. Obtained results showed that the highest level of all tested saponins (18.41 mg g^{-1} d.w.), expressed as the sum of protopanaxadiol and protopanaxatriol derivatives, was observed after 24 h of elicitation with 0.1 µM linalool (Fig. 1). The amount of detected ginsenosides was 29% higher than that observed in control samples. In comparison to untreated trials, raised saponin levels were also noted after using other linalool concentrations, except for 500 and 1000 µM. After 72 h, the impact of different linalool concentrations on saponin amount was also observed. However, metabolite levels were lower than those achieved during shorter elicitation. (Fig. 1).

Figure 2 illustrates changes in the protopanaxadiol (expressed as the sum of Rb1, Rc, Rb2, Rb3, and Rd) and protopanaxatriol (defined as the combination of Re and Rg1 contents) derivative contents in relation to linaloon concentrations. The results demonstrated that the same conditions, as observed for total studied saponins (0.1 M and 24 h elicitation), were also most favorable for accumulation of both the saponin groups. Initially, Rb group saponin content increased with increasing linalool concentration (0.01–0.1 µM) and achieved the value of 13.53 mg g^{-1} d.w. for 0.1 µM of linalool (Fig. 2). The level was 14.4% higher than the one noted for control samples. Furthermore, the rise of the elicitor amount in the medium (1–50 µM) slightly decreased Rb group saponin levels and contributed to inconsiderable fluctuations in their quantity. Linaloon concentration higher than 50 µM gradually decreased saponin content.

After application of 0.1 µM linalool and a 24-hour linalool treatment, saponins of the Rg group achieved the maximum level (4.89 mg g^{-1} d.w.) that was 2.3-fold higher in comparison to the untreated sample (Fig. 2). Other linalool concentrations (except for 1000 µM) and lengthening of elicitation time resulted in maintaining Rg saponin content at a similar level, i.e., higher than in control and below 4.89 mg g^{-1} d.w.

Moreover, levels of seven individual ginsenosides, including metabolites: Rc, Rb1, Rb2, Rb3, Rd, Rg1, and Re, were also established. Obtained results showed that shorter duration of elicitation positively affected accumulation of the majority of examined ginsenosides except for compound Rb1. A long-term linalool treatment did not increase the level of individual compounds above the maximum one, determined after 24 h of elicitation. However, a tendency for changes of tested saponin contents is similar for both treatment durations (Fig. 3). The most similar relationship of analyzed changes was observed for ginsenosides Re and Rg1. Presented findings revealed that application of 0.1 µM

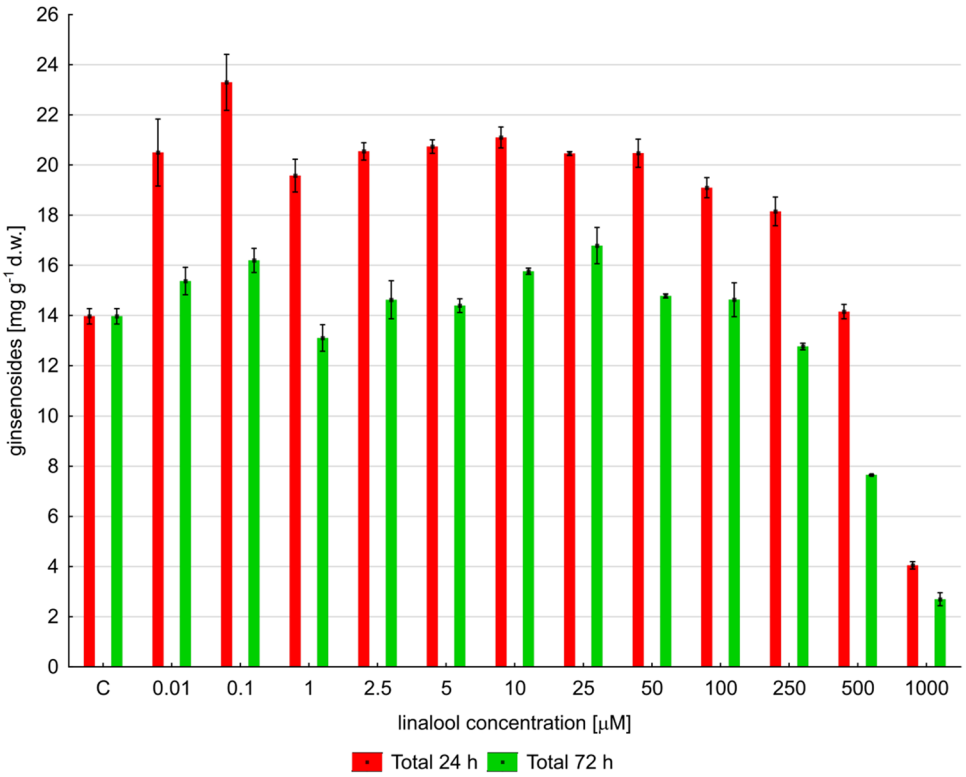

Fig. 1 Effect of linalool on ginsenoside level in *P. quinquefolium* hairy roots after 24- and 72 h elicitation

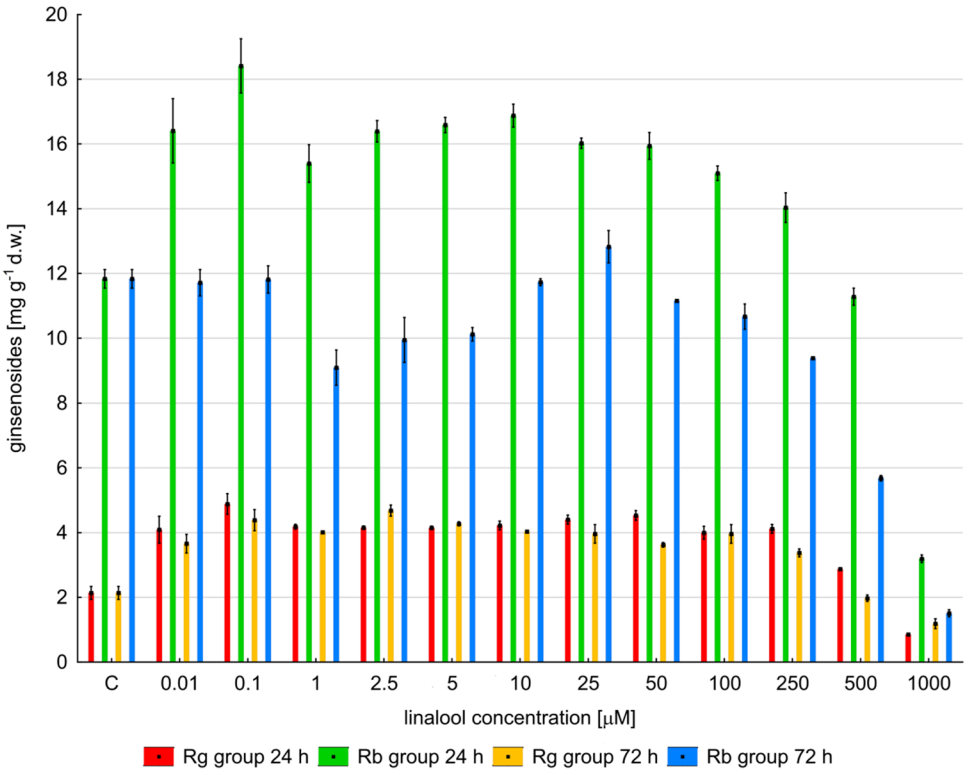

Fig. 2 Effect of linalool on Rb-group and Rg-group ginsenoside levels in *P. quinquefolium* hairy roots after 24- and 72 h elicitation

linalool proved the most effective strategy to enhance both Rg1 and Re accumulation (2.3-fold increase compared to control both for Rg1 and Re, Fig. 4).

Metabolites Rb2 and Rd reacted to linalool slightly differently than protopanaxartiol derivatives. The highest yield of these saponins was noted for the lowest applied linalool concentrations (0.01–0.1 µM, Figs. 3 and 4). Ginsenoside Rd demonstrated the strongest response among other tested protopanaxadiol derivatives (a 2.1-fold increase of Rd content relative to control, Figs. 3 and 4). Different results were observed for Rc and Rb3 ginsenosides. Initially, their content increased with an increasing amount of the elicitor. They reached the maximum level for higher linalool concentration (ranges: 5–10 and 2.5–25 µM linalool respectively for Rc and Rb3) than it was noted for Rb2, Rd saponins, or protopanaxatriol derivatives. Then, their content decreased. Different observations were obtained for Rb1 saponin. Linalool adversely affected this metabolite level - its amount was lower than in control regardless of linalool concentration and elicitation period (Figs. 3 and 4).

Discussion

The elicitation procedure is supposed to trigger a defensive reaction of a plant to various stress conditions. The main stimuli are elicitors, acting as molecules that induce signal transduction in a plant cell, which, in consequence, provokes a series of biochemical reactions leading to formation of many secondary metabolites (Ramirez-Estrada et al. 2016). Due to the fact that the elicitation process depends on many factors such as: type and concentration of the elicitor, stage of culture growth or time of exposure to the elicitor, conditions for the administration of exogenous elicitors in in vitro cultures must be appropriately selected for each culture and group of metabolites (Halder et al. 2019).

aEssential oils and their components are known for their multidirectional biological properties. Many reports prove their antimicrobial, antioxidant, anti-inflammatory, antioxidant, and anticarcinogenic activities (Elshafie and Camele 2017; Lima et al., 2018; Badea et al. 2019; Krzyśko-Łupicka et al. 2019; Patsilinakos et al. 2019; Spyridopoulou et al. 2019; Mancianti and Ebani 2020). The current study present the first examination of the application of linalool as a potential elicitor in enhancing ginsenoside production in hairy root cultures of *P. quinquefolium*. An effect of exposure times and different concentrations of linalool on the achieved ginseng saponin content in tested cultures was determined. This research showed that 24 h elicitation influenced accumulation of the majority of examined ginsenosides more positively than 72 h exposure time. Obtained results were consistent with those noted for trans – anethole, another essential oil ingredient that effectively stimulated ginseng saponin production also within a shorter period of time (24 h) (Kochan et al., 2018b). Furthermore, with regards

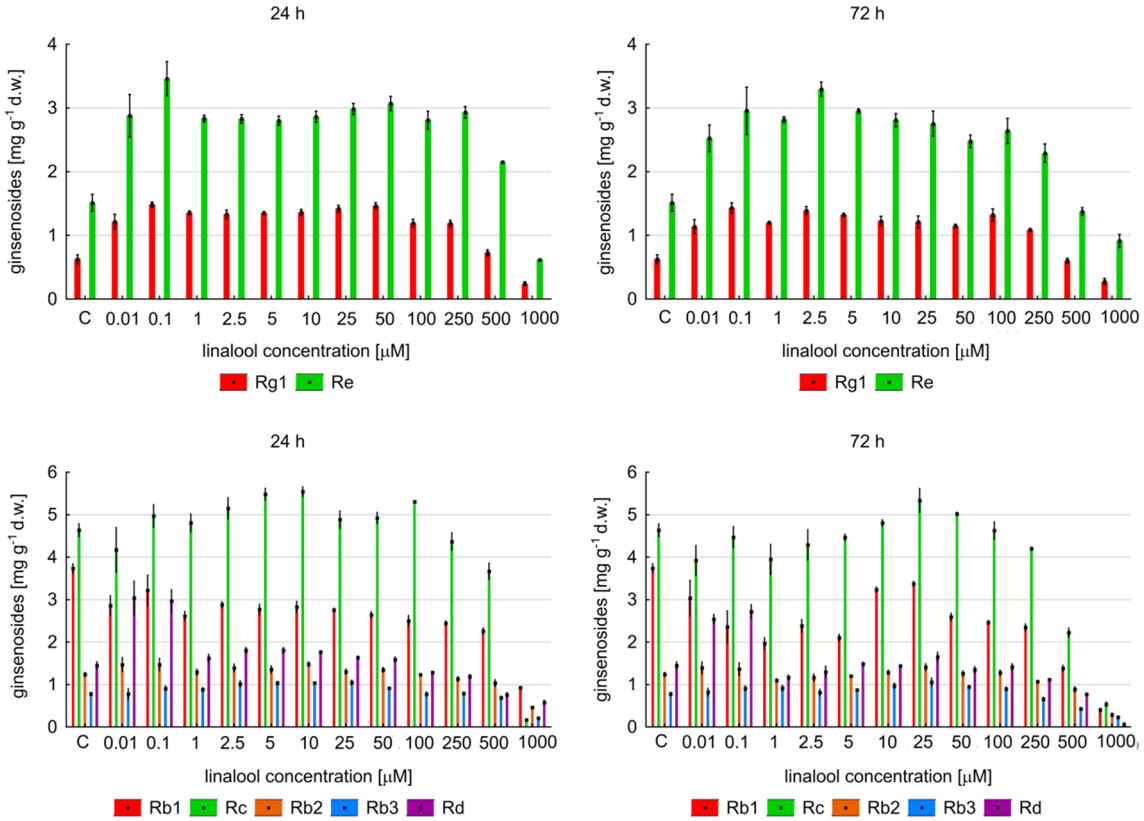

Fig. 3 Effect of linalool on the production of individual ginsenosides in *Panax quinquefolium* hairy roots after 24- and 72 h elicitation

to *P. quinquefolium* hairy roots, each of the applied elicitors required a different time to impact culture so that accumulation of studied metabolites could be efficient. The following elicitors required the given time: yeast extract—3 days, methyl jasmonate—7 days and abscisic acid—28 days (Kochan et al. 2017, 2018a, b, 2019). Other findings (e.g. described by Gai et al. 2019) regarding elicitated I*satis tinctoria* L. hairy root cultures, treated with salicylic acid, acetylsalicylic acid or methyl jasmonate and described by Wongicha et al. (2011) for hairy root cultures of *Glycyrrhiza inflata*, treated with chitosan, methyl jasmonate or yeast extract), confirm that optimal time of elicitor treatment can depend on both: the tested in vitro cultures and studies metabolites. Apart from the duration of exposure to the elicitor, there are two other parameters essential for the effectiveness of elicitation strategies. These are: specificity and concentration of elicitor. This study revealed that application of linalool at low concentration (0.1 µM) acted effectively on ginseng saponin content (29% increase in the level of ginsenosides in comparison to the control samples). Ginsenoside production was also enhanced through trans-anethole and methyl jasmonate (MeJa) treatment in *P. quinquefolium* hairy root culture but applied concentrations of these elicitors were significantly higher – 1 µM and 250 µM, respectively. In these conditions and after trans-anethole and methyl jasmonate elicitation, the yields of ginseng metabolites were 27.79 mg g^{-1} d.w. and 27.33 mg g^{-1} d.w (Kochan et al. 2018a, b). Research provided by other authors confirmed that application of MeJa increased the content of saponin in different ginseng culture in vitro, however MeJa concentration had to be high for example, 100 µM of methyl jasmonate was the most suitable concentration for ginsenoside production in *P. ginseng* adventitious root (Kim et al. 2004, 2009), while the values of 500 and 200 µM were optimal to intensify triterpene saponins in *P. ginseng* and *P. notoginseng* suspension cultures, respectively (Lu et al. 2001; Hu and Zhong 2008). Our study indicated that linalool can effectively enhance ginsenosideaccumulation in hairy root cultures of *P. quinquefolium* at significantly lower concentration (0.1 µM).

Results presented in this paper showed that the Rb group of ginsenosides dominated quantitatively over saponins belonging to the Rg group (Rb group/Rg group > 1) despite the elicitor concentration and its exposure time. This observation was found in other studies carried out on hairy roots of *P. quinquefolium* and in those that referred to *P. ginseng* adventitious root cultures (Kim et al. 2008; Marsik et al. 2014; Kochan et al. 2017, 2018a, b) or ginseng plant cultivated in fields (Kim et al. 2019).

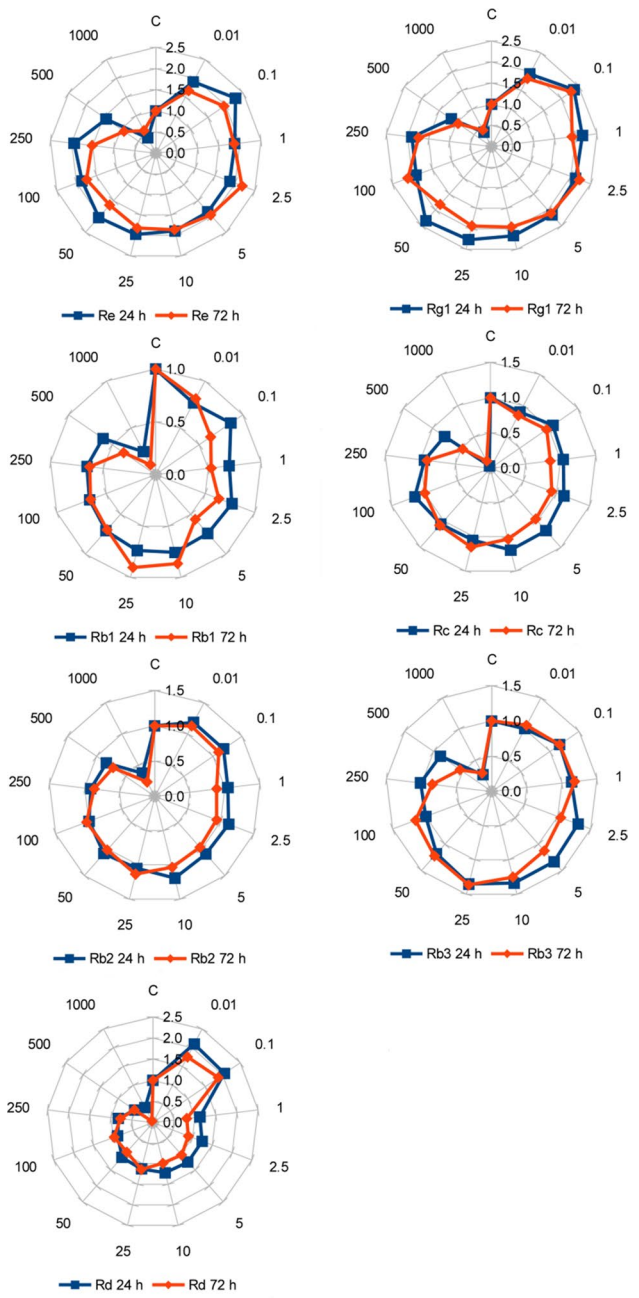

Fig. 4 Increase in individual ginsenoside contents in comparison to the control samples in *Panax quinquefolium* hairy roots after 24- and 72 h elicitation

An analysis of the effect of exogenous linalool indicated that different concentrations of linalool influenced individual ginsenoside accumulation in different ways. For example, the content of Rg1, Re and Rd metabolites doubled at 0.1 µM linalool in medium. Simultaneously, the level of Rb1 compound decreased below the level observed in untreated samples. The lowered Rb1 level was unexpected, especially in relation to previous research on the elicitation of hairy root *P. quinquefolium* cultures with trans-anethole, methyl jasmonate and yeast extract at concentration: 1µM, 250 µM and 50 mgL^{-1}, respectively. The aforementioned research showed a significant rise of Rb1 content (Kochan et al. 2017, 2018a, b). Similar observations for Rb1 ginsenoside were noted in studies on *P. ginseng* root cultures and *P. notoginseng* suspension cell cultures after elicitor treatment (Kim et al. 2004, 2009; Wang and Zhong 2002).

The increase in the amount of most studied saponins in *P. quinquefolius* hairy root culture under linalool treatment suggests that linalool could be used as an elicitor. Literature data indicated that cellular possible responses to elicitation are a complicated process. Elicitor molecules are recognized by specific receptors placed on the surface of plasma membrane or endomembrane. After that, suitable effectors are activated. Such effectors are ion channels, GTP bindings proteins (G-proteins) and protein kinases, and oxidative burst. They foster synthesis of signaling molecules, such as salicylic acid, jasmonic acid, or nitric oxide, which transfer the elicitor signals to defense genes. These genes are induced by elicitor treatment. Their expression induces enzymatic reactions that reprogram metabolic pathways and lead to secondary metabolite accumulation (Ramirez-Estrada et al. 2016; Shakya et al. 2019).

Glycosylation is the last stage of ginsenoside biosynthesis pathway. This process is very important in the production of ginseng saponins. Glycosylation is carried out by glycosyltransferases (GTs). GTs transfer glycosyl residues from activated sugars to ginsenoside aglycones, regulating formation of individual metabolites that differ in bioactivity, solubility, and stability glycosylases. So far not all ginseng glycosyltransferases have been known and characterized, so this biosynthesis step is not really clear. According to Li et al. (2022), metabolite Rd is converted to Rb1, Rb2, Rb3, and Rc with appropriate glycosyltransferases. This step of ginsenoside biosynthesis pathway could be likely blocked or hampered at the lowest linalool concentrations. In the effect, we obtained an overproduction of the Rd ingredient. However, it affected d efficient biosynthesis of ginsenoside Rb1, Rb2, Rb3, and Rc. Additionally, it was showed that compound Rg1 can be modified to Re saponin under suitable GT (Li et al. 2022). Results described in this study could be the ground for drawing a conclusion that glycosyltransferases leading to the formation of both metabolites were also upregulated under linalool. However, to confirm these hypotheses, further molecular investigations are necessary.

Conclusion

Our research, carried out in shake flasks, shows the effect of different linalool concentrations on the level of ginsenosides in *Panax quinquefolium* hairy root cultures. It also revealed

an effect of 24- and 72 h exposure time to this oil compound. Linalool can be applied as an elicitor which can increase triterpene saponin production in the studied cultures. The findings indicate that ginsenoside synthesis was intensified to a greater degree by elicitation for 24 h than 72 h.

The optimal concentration of linalool required for effective production of the studied ginsenosides and expressed as a sum of all examined metabolites was 0.1 µM. Besides, an optimal synthesis of Rg group saponins was observed in this linalool example. Individual ginsenosides belonging to protopanaxadiol derivatives demonstrated a varied response depending on linalool concentration. The highest content increase was noted for saponins Rd, Rg, and Re after 0.1 µM linalool treatment.

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Author contributions EK: conceptualization, supervision, methodology, lab work and results: examining hairy root cultures, obtaining extracts, writing—original draft preparation, review, and editing; GS: methodology, lab work, and ginsenoside content determination using HPLC, P. K.: statistical analysis, MS: writing—original draft, review, and editing. All authors read and approved the final manuscript.

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Declarations

Competing interest The authors have no relevant financial or non-financial interests to disclose.

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