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Activation effect of β-alanine and chitosan derivative on *A. glycyphyllos* and *A. membranaceus* seed germination and seedling growth and development

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Abstract. Agricultural cultivation of astragalus is fraught with a number of difficulties caused by both certain requirements for climatic conditions and individual characteristics of plants of this genus. In this study, carboxyalkylated derivative of chitosan was first proposed to use for improvement of astragalus propagation. Effects of N-(2-carboxyethyl)chitosan on *in vitro A. glycyphyllos* and *A. membranaceus* seed germination and seedling growth and development in comparing with β-alanine and chitosan acetate were detected. Carboxyethylation of chitosan leads to an increase in hydrophilic properties of the molecule, which enhances a penetration of nutrients inside the plant owing to improved solvating effect and bioadhesive activity. Seed germination assay were performed on Murashige-Skoog growth medium with or without tested compounds. N-2-Carboxyethylated derivative of chitosan was found to demonstrate active stimulating effect on the plant growth and development, contrary to the effect of acetate chitosan, but not to cause an activating effect on seed germination, while β-alanine does.

Key words: agricultural benefits, bioactivity, functional derivatives, morphological measurements, plant grow regulators, polysaccharides, statistical Friedman test, stimulating effect.

INTRODUCTION

Astragalus glycyphyllos L. (Bunge) and *Astragalus membranaceus L.* are nemoral plants, belonging to the *Fabaceae* family, which grow in a broad-leaved forest of the temperate zone. Astragalus is usually found in sparse oak, pine, deciduous and birch forests, steppificated meadow, in a steppe, on rocky hills and on sandy banks of rivers. These amnicolous plants grow in Europe, East Asia (Northern Mongolia (*A. membranaceus*), Korea, Northeast, North and Northwest China), Western (*A. glycyphyllos*) and Eastern (*A. membranaceus*) Siberia, the Far East (World Health

Organization. Regional Office for the Western Pacific, 1998; Podlech, 2008; Podlech & Zarre, 2013). Sub-acid to alkaline, loam and sandy-loam soils favor the growth of them (Liu et al., 2016; Yang et al., 2020). The loss, alteration, and degradation of habitats from recreational load, livestock grazing and trampling, residential, commercial, and industrial development, forest fires and colonization by trees and shrubs are limiting factors contributing to threatened status of *A. glycyphyllos* species. The potential danger to *A. membranaceus* is low temperatures in the range of 0–10 °C, which have negative effects on the development of the plants in China and Europe and make population to decrease in size and density (Liu et al., 2019). At the same time, high temperatures (30–40 °C) cause oxidative stress, accumulation of reactive oxygen species, photosynthetic disorders and therefore limit the growth of seedlings (Zhou et al., 2012; Chopra et al., 2017; Laxa et al., 2019). Finally, human intervention transforms the established evolutionary relationships among species and results in their degradation (Zhuravlev et al., 1999).

Biologically active components of *A. glycyphyllos* and *A. membranaceus* species are of great importance for prophylaxis and therapy, and their efficient extraction and alternative production are important biotechnological task due to the high harvest pressure on wild populations (Bratkov et al., 2016). Extracts of leaves and roots are used in the treatment of neurological disorders, dermatitis, rheumatism, dysentery, venereal, gastrointestinal and acute respiratory diseases (Godevac et al., 2008), as well as in body weight reduction due to their antidiabetic properties (Shen et al., 2006; Ng et al., 2014). Diuretic properties make their application in kidney disease and gout possible (Guarino, De Simone & Santoro, 2008).

Secondary metabolites of the flavonoid class, especially formononetin and calicosin, exhibit strong antioxidant properties owing to the presence of hydroxyl groups on the polyphenol ring (Shirataki et al., 1997; Toda & Shirataki, 1999; Bratkov et al., 2016). At the same time, polyphenols have a detrimental effect on tumor cells, acting as initiators of caspase-dependent apoptosis and inhibitors of anti-apoptotic proteins of the mitochondrial membrane (Auyeung & Ko, 2010). Along with that, formononetin and calycosin suppress the growth of human pathogenic intestinal bacteria (Zhang et al., 2014), and also exhibit antifibrotic, anti-inflammatory and hepatoprotective properties (Wang et al., 2001; Huh et al., 2010). It is significant that, the only flavone apigenin is contained in extracts of many plant species of the genus *Astragalus* (Pistelli, 2002) and possesses the entire spectrum of bioactive properties of individual flavonoids (Salehi et al., 2019).

Carbohydrates such as α-1,4(1,6)-glucan, arabinose-galactose, rhamnose-galacturonic acid polysaccharide and arabinose-galactoprotein one isolated from *A. glycyphyllos* and *A. membranaceus* are used not only in chemical, but also in supportive anticancer therapy owing to reduction the negative effect of radiation therapy (Wang et al., 2018). The immunoregulatory properties of the polysaccharides are due to the increased functioning of immunocompetent organs and cells and the associated effects include but not limited to antitumor, anti-inflammatory and antiviral ones (Jin et al., 2014; Wang et al., 2018).

Triterpenoid sapogenins, triterpenes and sterols are constituents of various modern drugs (Chandler, 1985; Badam, 1997; Pistelli, 2002; Salari et al., 2003; Yu et al., 2013). A distinctive feature of phytosterols is the ability to act as precursors of hormones (sex and corticoid) (Sundararaman & Djerassi, 1977) and vitamins (for example, D3)

(Kametani & Furuyama, 1987), as well as to reduce cholesterol levels (Choudhary & Tran, 2011). Although against all the above mentioned, one of the most significant substances of plants of the genus *Astragalus* remains the triterpenoid saponin, astragaloside IV (Zhang et al., 2006, 2020; Liu et al., 2017), since its derivative cycloastragenol is an effective geroprotector which activates the telomerase enzyme (de Jesus et al., 2011; Ip et al., 2014).

The polyaminosaccharide chitosan and its derivatives are widely studied for their application in plant growing (El Hadrami et al., 2010; Ferri & Tassoni, 2011; du Jardin, 2015; Xing et al., 2015; Malerba & Cerana, 2016; Rafiee et al., 2016). In addition to regulation of growth and development, their use, both individually and in combination with other preparations, leads to the improvement of plant resistance to pathogens and abiotic stress conditions (du Jardin, 2015; Faoro & Gozzo, 2015; Malerba & Cerana, 2016; Rafiee et al., 2016; Dawood, 2018; Sailo et al., 2018; Kolesnikov et al., 2020). The nature of these defence responses depends on the plant hosts, the stage of growth and development, the physicochemical properties, the composition and structure of applied chitosans (Faoro & Gozzo, 2015). Despite chitosan is a well-known biostimulator used to wide variety of food crops there are only a few publications dealing with medicinal plants, to our knowledge. To the best of our knowledge, there is only one publication on an elicitation practice using chitosan to *Astragalus* plants, namely, elicitation protocol for the production of the *A. membranaceus* hairy root cultures with enhanced level of formononetin and calycosin (Gai et al., 2019). Chitosan derivatives in this regard have not been previously studied.

When added in the composition of growth media, amino acids have been known to initiate and accelerate seedling growth. Thus, for example, the sprout formation processes are known to be accelerated in the meristematic zone when growing the seedlings of *Peltogyne purpurea* with the use of L-cysteine (Esteban et al., 2015).

Considering the abovementioned and having regard to the increased solubility of carboxyalkyl chitosans compared with the native chitosan (Bratskaya et al., 2009), we combined the chitosan core and the amino acid (β-alanine) moiety in one molecule. Such a modification of chitosan leads not only to an improvement in solubility, but also allows us to expect an increase in bioavailability, hydrophilic properties and, as a consequence, in solvating effect and bioadhesive activity, which promotes the conversion of bio-unavailable nutrients to the more accessible forms, suitable for the involvement of such compounds in metabolism. This properties are favourable to plant growth and development biostimulation. In this work, comparative study of N-(2-carboxyethyl)chitosan, β-alanine and chitosan acetate effects on *A. glycyphyllos* and *A. membranaceus* seed germination and seedling growth and development were performed *in vitro*.

MATERIALS AND METHODS

Plant materials

Mature *A. glycyphyllos* and *A. membranaceus* seeds, showing a germination percentage of 98% in the laboratory conditions, were used for studying the biological activity. All the seeds were harvested from wild habitats and stored at 4 °C prior to the experiments.

Feedstock and chemicals

N-(2-carboxyethyl)chitosan with the degree of substitution 1 (N-CECS), chitosan acetate (CA), and β-alanine (BA) were used for bioactivity evaluation. N-CECS was synthesized according to the previously described procedure using the 'synthesis in gel' method (Pestov, Zhuravlev & Yatluk, 2007). Physicochemical characteristics were found to be in accordance with the data published previously.

Chitosan were purchased from Bioprogress and used as starting material in the synthesis route. Chitosan acetate was synthesized (the degree of acetylation (DA) 0.18; 500 kDa; the ash content 0.16%) by dissolving the chitosan in the equivalent amount of 3% aqueous acetic acid solution with subsequent precipitation, filtration and drying at 25 °C. β-Alanine (98%) produced by AlfaAesar was used with no additional purification required. Premixed medium powder containing basal salts and vitamins of Murashige-Skoog medium were purchased from BioloT.

Seed germination

The seeds (30 seeds for each replicate) were sterilized in the following steps: 1. dipping in ethanol solution (70%) for two minutes, 2. immersion in a freshly prepared sodium hypochlorite (2.5%) at room temperature for 15 minutes, 3. rinsing with sterile distilled water three times for 10 minutes. The seeds were mechanically scarified using a sterile dissecting needle to break the exogenous physical dormancy. The seeds were sown on Murashige-Skoog growth medium (Murashige & Skoog, 1962) (1 × MS salts, 30 g L^{-1} sucrose, and 10 g L^{-1} agar, pH 5.7) with or without various tested compounds and then incubated in a growth chamber with a 16 h light photoperiod at 23 ± 2 °C. The studied compounds were added to the growth medium at a concentration of 100 mL g^{-1}. The medium without growth regulators served as control. The number of germinated seeds was recorded 5, 10 and 15 days after being sown on the medium. Duration of passages varied within 4–8 weeks. Radicle emergence of > 1 mm indicated seed germination.

Statistical analysis

Three replicates were used for each treatment and results of morphological measurements are presented as the mean values together with the standard errors. *A p* value of less than 0.05 was considered to show a statistically significant result. As the data distribution was not normal, the non-parametric statistical Friedman test (Friedman, 1937) was used for assessment the statistical significance of the effects caused by adding the tested compounds to the growth medium. For statistical processing of the qualitative data obtained, we introduced the following simple rating scale (Table 1). Statistical analyses were done using Statistica 10.0 portable software.

Table 1. Evaluation scale of the plant growth and development

Score	Development stage
1	seed germination start
2	first true leaf stage
3	apparent isolation of taproots
4	appearance of lateral roots
5	formation of a mature plant in the vegetative phase

RESULTS AND DISCUSSION

Germination, growth and development. The efficiency of chitosan application has been demonstrated for a variety of plants, including species of the genus *Astragalus* (El Hadrami et al., 2010; Ferri & Tassoni, 2011; Farouk & Amany, 2012; Mondal et al., 2012, 2013; Yin et al., 2012; Xing et al., 2015; Malerba & Cerana, 2016; Phothi & Theerakarunwong, 2017). Besides, the use of chitosan has been reported to promote accumulation of phytoalexins in *A. membranaceus* (Gai et al., 2019). Considering the known literature data, germination of the *Astragalus* seeds in the present study was initially carried out in growth media containing N-(2-carboxyethyl)chitosan or chitosan acetate or β-alanine.

Assessment of germination was commenced five days after being sown on the medium. A seed was considered germinated when the emerged radicle had reached a length greater than two length of the seed.

The total germination and seedling growth rate of *A. glycyphyllos* were considerably greater than those of *A. membranaceus*, which is probably associated with the seed storage conditions of the latter. According to the literature data, contamination of the seeds with mold fungi and putrefaction bacteria results in decreased germination energy and lowered germinability of *Astragalus* spp. (Galtsova & Silantieva, 2015). Most *Fabaceae* species are known for their hardseededness which is the cause of seed dormancy. So, germinating ability is usually low without scarification (Long et al., 2012; Statwick, 2016) and such treatment is used for overcoming the hardseededness and significant improving of the germination rate of *Astragalus* seeds (by 17–52%).

The seed germination start for *A. glycyphyllos* was observed on day 8 in the three different media (germination percentage lies in the range 9.09 to 16.7%, Table 2). Seedlings in the control line showed radicles much longer than those in the other ones.

Table 2. Germination percentage of *A. glycyphyllos* (*A. g.*) and *A. membranaceus* (*A. m.*) seeds in the growth media upon addition of various compounds

Days	Germination percentage							
	N-CECS		CA		Control		BA	
	A. m.	*A. g.*	*A. m.*	*A. g.*	*A. m.*	*A. g.*	*A. m.*	*A. g.*
5	0	0	0	0	0	0	0	0
8	0	9.1 ± 2.7	0	0	25.0 ± 4.4	12.5 ± 3.2	0	16.7 ± 4.6
12	0	27.3 ± 3.4	0	12.5 ± 3.6	25.0 ± 4.4	37.5 ± 4.1	0	25.0 ± 5.9
15	0	45.5 ± 7.5	0	37.5 ± 7.1	25.0 ± 4.4	75.0 ± 10.5	0	25.0 ± 5.9
22	0	54.6 ± 9.2	0	37.5 ± 7.1	50.0 ± 6.2	75.0 ± 10.5	0	25.0 ± 5.9
26	25.0 ± 5.3	54.6 ± 9.2	0	37.5 ± 7.1	50.0 ± 6.2	75.0 ± 10.5	0	33.3 ± 6.3
35	25.0 ± 5.3	54.6 ± 9.2	0	50.0 ± 8.9	50.0 ± 6.2	100.0 ± 11.2	0	33.3 ± 6.3
47	25.0 ± 5.3	63.7 ± 9.8	0	50.0 ± 8.9	50.0 ± 6.2	100.0 ± 11.2	0	33.3 ± 6.3

By day 12, the control seedlings had the first true leaf appeared, and the main root system was observed to be coming to the branching stage. To our knowledge, during the *A. glycyphyllos* ontogenesis process, radicle shows elongation on day 8 or 9, and the first true leaf develops 17–20 days after being planted. In the other media, cotyledons were observed to have appeared. In our experiments, the worst growth scenario was realized for the medium with CA added.

On day 15 of the experiment, the growing processes were observed to accelerate in case of the N-CECS-containing medium, where lateral roots appeared. Whereas the seedlings in the BA and CA-containing media continued to be inferior to the N-CECS-containing one in their growth and development rates.

By day 22, seedlings in the BA line showed considerable growth of the vegetative part of the plants with retarded growth of the root system; and the total lack of root development was observed in a number of cases. All of the plants in the CA line manifested the signs of considerable growth and development inhibition: the colorlessness (lack of photosynthetic chloroplasts), the lack of root system development, and the emerged cotyledons not taking the vertical position but lying down on the medium. This seems contrary to the literature data on a positive effect of chitosan on the growth of hairy root cultures of *A. membranaceus* and phytoalexins accumulation, which has been observed to be especially pronounced 18–24 hours after treatment depending on chitosan concentration (Gai et al., 2019). Good growth and development of the plants were observed in two media: the control medium and the N-CECS-containing one. Plants in the N-CECS was characterized by a significant both stems and petioles elongation.

By day 26, the young plants had been keeping the growth rate to be a constant in two line: the control and N-CECS-containing media. In the cases of CA and BA-containing media, the tested compounds apparently inhibited the growth processes: development of the root system was either not observed or seen with abnormalities; true leaves were either not developed or their growth was considerably retarded.

On day 35, grown in the N-CECS-containing and control media, the young plants were found to have been formed completely, i.e., they exhibited well-branched and proportionally developed root systems and clearly seen short shoots with four to five true leaves. In the case of BA-containing medium, the young plants were characterized by formation of short shoots having fewer than or equal to three leaves, and development of the root system was still retarded considerably. In the CA-containing medium, the root system failed to develop, a portion of the seedlings showed true leaves, and such plants demonstrated vertical position.

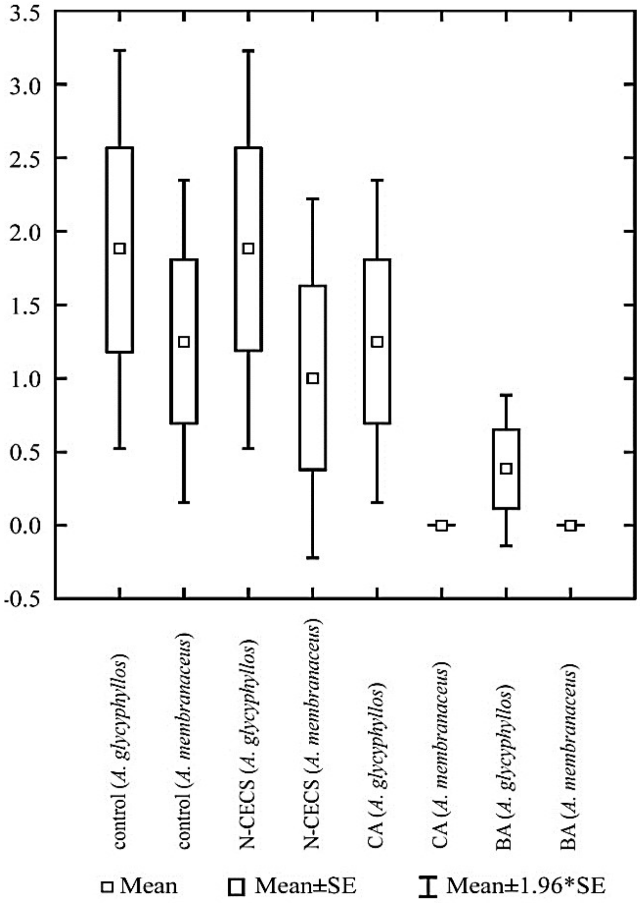


Figure 1. Average growth patterns for *A. glycyphyllos* and *A. membranaceus* plants. The growth patterns were obtained on the basis of the rating scale for the major qualitative growth parameters.

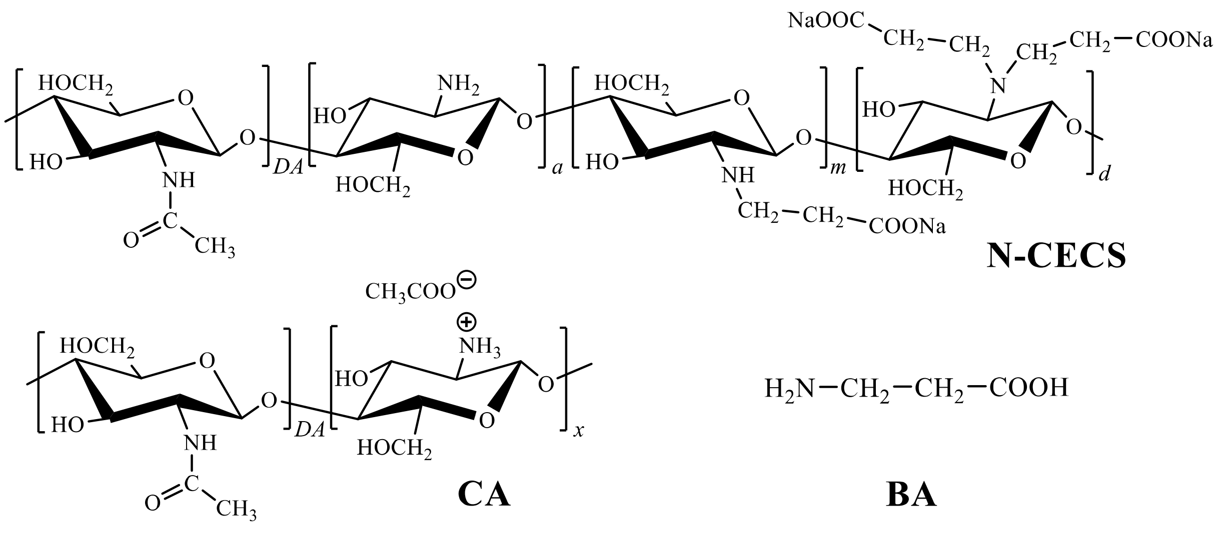
At the initial stages of germination, a 3 days delay observed in the control medium for *A. glycyphyllos* and a 21 days delay observed in the N-CECS-containing medium for *A. membranaceus* were considered as the most notable differences in the comparative analysis of the germination dynamic of the seeds.

The maximum germination rate was observed for the *A. glycyphyllos* seeds in the control medium. High germination rates were also noted for the mentioned seeds planted in the growth medium containing N-CECS; however, by day 47, the germination percentage there was found to be somewhat lower in comparing with the control medium.

The worst germination percentage (33.3%) of the *A. glycyphyllos* seeds were pointed out in the BA-containing medium, although, by day 5, the corresponding value had been the highest (16.7%) among all the media studied.

The statistical analysis of the obtained data was performed using the Friedman test and demonstrated that all the tested compounds cause a significant effect on the growth activation and all other factors and processes involved in mature plant formation (growth rate, development of vegetative and root systems). A growth pattern closest to the control was displayed by *A. glycyphyllos* plants exposed to N-CECS stimulation (Fig. 1). The patterns of *A. membranaceus* plants differ quite significantly between the lines; however, the general positive dynamics of stimulation are observed in the N-CECS and the control lines.

Evaluation the effect caused by the compounds. Contrary to the known efficacy of using chitosan to a variety of living organisms from single-celled to many-celled ones, including *Astragalus* plants (El Hadrami et al., 2010; Ferri & Tassoni, 2011; Farouk & Amany, 2012; Mondal et al., 2012, 2013; Yin et al., 2012; Xing et al., 2015; Malerba & Cerana, 2016; Phothi & Theerakarunwong, 2017), the present study demonstrates water soluble chitosan acetate to inhibit the growth and development of the plants, while its carboxyethylated derivative is shown to activate the growth processes. Considering N-CECS as N-derivative of β-alanine (Scheme 1), this amino acid was used as an additional reference compound and demonstrated a somewhat activating effect but only during the first eight days of the experiment.



Scheme 1. Structure of N-(2-carboxyethyl)chitosan with the degree of substitution 1 (N-CECS), of chitosan acetate (CA) and β-alanine (BA).

Generally, it should be suggested that β-alanine, being a low-molecular amino acid, causes an activating effect due to its ability penetrate the seed tissue through the seed coat. Despite the presence of the β-alanine fragment in the N-CECS structure, the latter does not cause such an activating effect because of inability to penetrate the tissues of germinated seeds. Therefore, the mechanism underlying the growth-regulating effect of chitosan and N-CECS is caused exclusively by an external interaction between macromolecules and tissues of a developing plant. The fundamentally different activities of acetate chitosan and N-CECS revealed in this study could result from different values of the hydrophilic-lipophilic balance. The presence of carboxyl groups in the molecule of N-CECS provides the greater hydration degree and, as a consequence, the greater solvating effect and bioadhesive activity as compared to CA.

CONCLUSIONS

Search and development of new fertilizers, sbiostimulators and plant protection agents based on safe and non-toxic organic compounds is agrochemistry frontiers in agricultural sustainability. Organic design and synthesis feasibilities allows for obtaining such compounds. Bio-based molecules and polysaccharides primarily have great advantages stemming from huge availability of feedstocks from large-scale synthesis wastes. Chitosan, being polymer of natural origin with increased reactivity in comparing with cellulose or starch, is currently commercially-available polysaccharide with boundless opportunities for development of new eco-friendly agrochemicals. Its synthetic flexibility offers the prospect of the design and construction of new plant protection agents and growth regulators with agricultural benefits.

The present work is focused on the mentioned research trend in so far as native chitosan is limited in use to agriculture due to, among other things, its limited solubility and a narrow spectrum of bioactivity. At the same time functional derivatives of chitosan expand the spectrum of both compounds/materials and biological effects. Using *A. glycyphyllos* and *A. membranaceus* as examples, it was found that N-2-carboxyethylated derivative of chitosan do demonstrate active stimulating effect on the plant growth and development, contrary to the acetate chitosan. Despite the presence of the β-alanine fragment in the N-CECS structure, the latter does not cause an activating effect on seed germination, while β-alanine does.

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