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Effect of rutin-ammonium complex on the physiological state of Scots pine seedlings

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Abstract. In the plant organism, phenolic compounds have a non-specific effect on the processes of morphogenesis and perform a wide range of regulatory and protective functions. Of particular interest are the processes associated with the complex formation of flavonoids as a result of their interaction with ammonium forms of nitrogen. Polar compounds that are formed in tissues by chemical transformation are quite mobile in soil solutions and exhibit high biological activity. The properties of phenol-ammonium complexes are of considerable interest in terms of morphogenesis, physiology of resistance, and in the system of interaction of plants with soil microorganisms. This is also extremely important in the production of high-quality planting material that is resistant to unfavourable factors. The effect of the phenol-ammonium complex was studied on seeds and seedlings of Scots pine. Quantitative indicators of germination energy and similarity were determined by the seed germination method. Biochemical profiling of seedling tissue extracts was performed by high-performance thin-layer chromatography. It has been experimentally confirmed that rutin (quercetin-3-o-rutinoside) after interaction with a 10% aqueous solution of ammonia forms a complex of substances, among which polar products were detected by chromatography, which potentially affect growth regulation. At a total concentration of 15 mg/l, these substances significantly increased germination energy and germinating ability. In pine seedlings, they stimulated the growth of roots and shoots. The effect of the complex of organic compounds on seedlings depended on the concentration, duration of seed treatment, and had a prolonged effect. The resulting phenol-ammonium complex at a concentration of 10-15 mg/l contributed to an increase in the amount of chlorophylls and carotenoids in the tissues of seedlings, and at 20-40 mg/l it increased the content of phenolic synthesis products

Keywords: rutin, flavonoids, ammonium, growth regulator, Scots pine, seeds

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Introduction

Phenolic compounds are one of the important components of the synthesis of secondary metabolites in the plant body (Lattanzio, 2013). The functions of phenolic compounds are extremely diverse and require a comprehensive study (Buer *et al.*, 2010; Caretto *et al.*, 2015). Plant phenols perform mechanical, structural and signalling functions, protect tissues from UV, perform the functions of antioxidants, chelate transition metal ions, remove radioactive elements from the body, protect the plant organism from the effects of adverse factors, pests, and pathogens (Blokhina *et al.*, 2003; Naikoo *et al.*, 2019; Tegelberg *et al.*, 2001; Zagoskina & Nazarenko, 2016; Winkel-Shirley, 2002).

When obtaining planting material, considerable attention is now paid to the investigation of physiological reactions of plants to the action of biologically active substances, organic and mineral fertilisers, which are used to improve the quality of planting material and increase plant resistance to adverse factors (Kovalevsky & Taranenko, 2013; Pinchuk, *et al.*, 2017; Pinchuk & Likhanov, 2018).

The secondary metabolism of most higher plants is aimed at the active synthesis of phenolic compounds. However, the concentration of different classes of phenols in plant tissues may differ significantly. In particular, the amount of phenol carboxylic acids in the leaves correlates with the concentrations of phytohormones. This is conditioned by the fact that oxycoric and oxybenzoic acids have a non-specific effect on morphogenesis processes (Santelia, 2008; Volynec, 2013) and perform a wide range of regulatory and protective functions (Zaprometov, 1993; Grana *et al.*, 2017). To understand the role of individual oxycoric and oxybenzoic acids, as well as flavonoids, it is of great interest to model the conditions of their individual and complex effects on the plant organism. In addition, important but poorly understood processes are those associated with the complexation of phenolic compounds, in particular flavonoids, in plant tissues and the rhizosphere during their entry into the soil (Grodzinsky, 1973; Boyer *et al.*, 1989).

Phenolic compounds are actively secreted by physiologically active root zones. Some flavonoids (in particular, quercetin and its glycosides) have high reactivity to ammonium cations (Likhanov *et al.*, 2021). Polar compounds that are established as a result of chemical transformation of polyphenols are quite mobile in soil solutions and exhibit high biological activity (Melnychuk *et al.*, 2011). Their effect on growth processes, development of root system, synthesis of plastid pigments and secondary metabolic products is already observed at a concentration of 20 μg/ml. A similar effect, for example, was confirmed for kaempferol-3-O-β-D-glucoside. An increase in its concentration from 15 μg/ml and above increased the elongation of shoots of *Echinochloa colonum* L. (Li Jun, 2011). Current research also confirms that invasive plants *Triadica sebifera* (L.) Small are characterised by a high content of quercetin in root exudates. It is assumed that in the composition of exudates, this flavonol is a key in the signalling interactions of plants with mycorrhizal fungi and other soil microorganisms (Anand *et al.*, 2016; Tian, 2021).

Since the biological activity of compounds formed as a result of the interaction of flavonols with ammonium forms of nitrogen has not been sufficiently investigated, the purpose of this study was to consider the effect of the rutin-ammonium complex on the physiological state of Scots pine seedlings.

Materials and Methods

Preparation of polar complexes based on rutin with an aqueous solution of ammonia. A test tube containing 10 mg of rutin (Merck, Germany) was stirred on a vortexer in 300 μL of distilled water for 60 s. Then 100 μL of a 10% aqueous ammonia solution was added and stirred for 30-40 seconds until rutin was completely dissolved. The resulting solution acquired a rich brown colour. The volume of the solution was adjusted with bidistilled water to 1 ml and stored at +4°C. The resulting stock solution contained 10 mg/mL of polar compounds. To investigate the effect of the rutin-ammonium complex on seeds and seedlings of Scots pine, the initial solution was diluted with distilled water. Seeds were soaked in prepared solutions (concentrations – 10, 15, 20, and 40 μg/ml) for 3 and 24 hours before germination.

Method for determining the germination energy and germinating ability of Scots pine. Determination of germination energy and germinating ability of Scots pine was carried out according to DSTU 8558:2015 "Seeds of trees and shrubs. Methods for determining seed qualities (germinating ability, viability, good quality)" (SSU, 2015). Seed germination was performed in Jacobsen apparatuses with a photoperiod of 12 hours at a constant temperature (22±2°C). Seeds of Scots pine, prepared for germination, were laid out on a bed of 100 seeds, not allowing touching each other, in order to avoid transmission of infection from diseased to healthy seeds. After decomposition of seeds of one medium sample, the work surface, bed, cap and tweezers were disinfected with alcohol. To determine the indicators of germination energy and germinating ability, 4 samples were laid in triplicate. Evaluation and accounting of sprouted seeds were carried out on days 5, 7, 10, and 14. Determination of germination energy for Scots pine seeds was carried out on Day 7, germinating ability – on Day 14.

High-performance thin-layer chromatography methods. Biochemical profiling of extracts from Scots pine seedling tissues was performed by the HPTLC method on silica gel G60 plates (Merck, Germany).

Flavonoid separation was performed in solvent systems: ethylmethylketone-ethyl acetate–methanol–water (v/v/v/v – 30:20:5:5); ethyl acetate-formic acid–acetic acid–water (v/v/v/v – 100:11:11:25). To determine the chemical nature of the substances, the chromatograms were treated with 0.5% NP reagent and 1% PEG 400 followed by heating (5 min at 105°C).

Separation of chlorophylls, carotenoids, and xanthophylls was performed in the system: toluene–ethyl acetate–formic acid (v/v/v – 2:6:1). Visualisation of phenolic compounds and plastid pigments was performed in UV for λ_{max} =365 Nm.

The mobility coefficients of individual compounds (Rf) were determined photodensitometrically using the Sorbfil TLC Videodensitometer software suite ver. 2.3.0.2994 (JSC Sorbopolymer, RF).

Statistical data processing. The obtained data were presented as an average value ± standard error (X±SE). The validity of differences between the mean values ($p<0.05$) was determined by the method of univariate analysis of variance (one-way ANOVA) and Tukey's honest significance test (HSD) in the XLSTAT software suite (Addinsoft Inc., USA, 2010).

Results and Discussion

It has been experimentally confirmed that rutin (quercetin-3-o-rutinoside) after interaction with a 10% aqueous solution of ammonia forms a complex of substances that contain products that play an important role in regulating the processes of plant growth and development. Chromatographic studies have confirmed that after the interaction of rutin with an aqueous solution of ammonia, 5 new products are formed, which, according to the mobility coefficients (Rf), belong to midpolar and polar substances (Fig. 1).

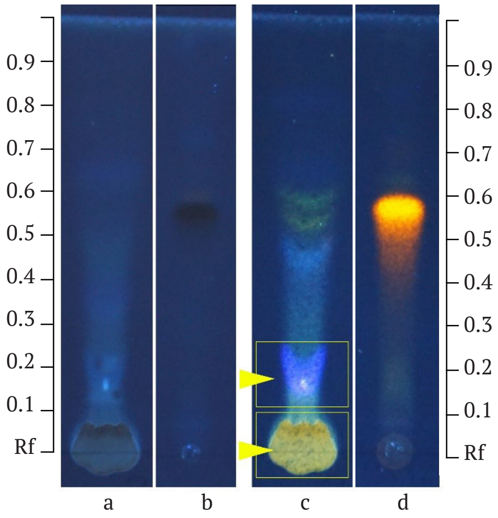

Figure 1. Chromatogram of the rutin-ammonium complex before (*a*) and after (*c*) treatment with NP reagent; standard rutin solution before (*b*) and after (*d*) processing; arrows indicate bioactive candidate substances

After 72 hours, no rutin was detected in the resulting solution. Thus, over time, a complete biochemical transformation of flavonol occurred. On the chromatogram, after treatment with NP reagent, two products with Rf ~0.53 and 0.58 with flavonol–characteristic UV fluorescence (λ=365 Nm) were detected in small amounts. The highly polar compound with bright blue fluorescence with an Rf of ~0.18 and the substance that remained on the starting line were fairly stable. This gives grounds to consider them as candidate substances with high biological activity and the ability to influence the growth processes of plants. The effect depends on the concentration of the rutin-ammonium complex solution. Prolonged exposure indicates the ability of water-soluble polar compounds, which are part of the phenol-ammonium complex, to penetrate through the seed shells into living tissues, remain stable in them and further participate in the regulation of root growth.

In particular, the pretreatment of Scots pine seeds with various dilutions of the initial solution of the rutin-ammonium complex showed that at a concentration of 10 μg/ml, the indicators of germination energy and germinating ability do not significantly differ from the control (Table 1).

Table 1. Effect of rutin-ammonium complex on germination energy and germinating ability of Scots pine (n=3, X±SE)

Concentration, μg/ml	Germination energy, %	Germinating ability, %
0 (control)	58.0±1.73	69.7±1.45
10	60.3±0.88	72.0±1.15
15	67.7±1.45**	78.0±1.73*
20	64.0±0.59*	72.3±1.45
40	52.0±1.15*	63.7±2.03

Note: the significance of differences was evaluated using univariate analysis of variance and Tukey's honest significance test (HSD); * – the difference with the control is significant for $p < 0.05$, ** – for $p < 0.01$

When the concentration of the complex of active substances in the solution increases to 15 and 20 μg/ml, these indicators increase, but significantly decrease when the concentration reaches 40 μg/ml. Further observations of the processes of germination and seedling development revealed the prolonged effect of organic compounds (Fig. 2).

Figure 2. Seedlings of Scots pine after treatment of seeds with rutin-ammonium complex (*1* – 2 hours; *2* – 24 hours); ruler – 20 mm; *a* – control; *b*, *c*, *d*, *e* – sprouts that were pretreated with solutions with concentrations of 10, 15, 20, and 40 µg/ml, respectively

Soaking the seeds in solutions with different concentrations for 24 hours stimulated the growth of seedlings. At concentrations of 10 and 20 µg/ml, the growth processes of stems and cotyledons were more sensitive to bioactive substances (Fig. 3, *a*).

Under conditions of increasing concentration of phenolic compounds, the growth rate of stems slowed down, and roots, on the contrary, increased (Fig. 3, *b*). The difference in the growth processes of roots and shoots indicates the presence of a certain tissue specificity in the plant body, which is conditioned by the anatomical structure and functions of aboveground and underground organs.

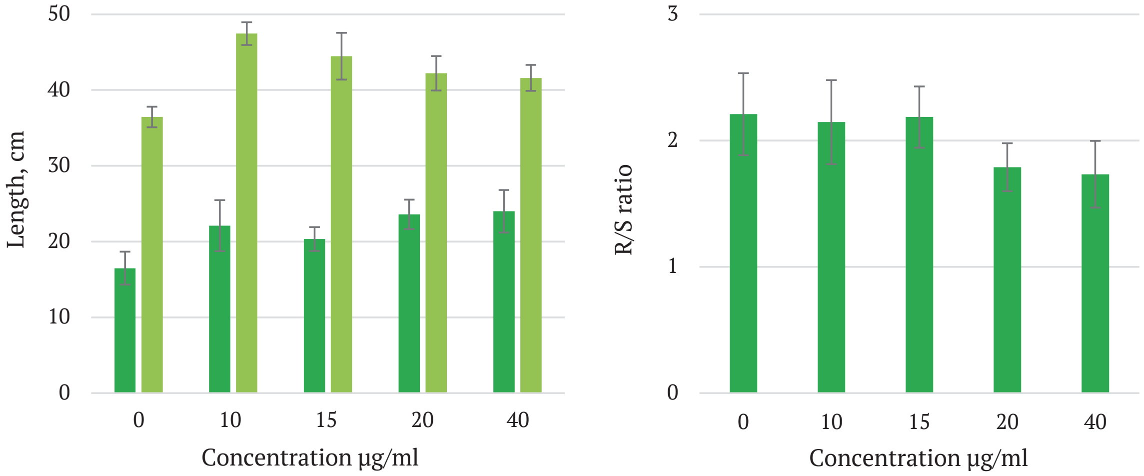

Figure 3. Effect of rutin-ammonium complex on root and shoot growth rates (*a*) and their relationship (*b*) in seedlings of Scots pine

Stimulating root system development is an important element of a plant survival strategy in the face of fierce competition for spatial resources and nutrients, especially in conditions of moisture deficiency. For adventitious species with a wide range of adaptive responses, active root system development is one of the main strategic features that ensure the successful naturalisation of plants in new conditions.

A significant influence of the rutin-ammonium complex on the qualitative composition of photosynthetic pigments included in light-harvesting complexes and auxiliary pigments – xanthophylls and carotenoids – was also revealed. In Scots pine seedlings that were treated with a solution with concentrations of 10 and 15 µg/ml, the total amount of plastid pigments increased (Fig. 4, *a*, *c*, *d*). The amount of chlorophylls in a and b also increased.

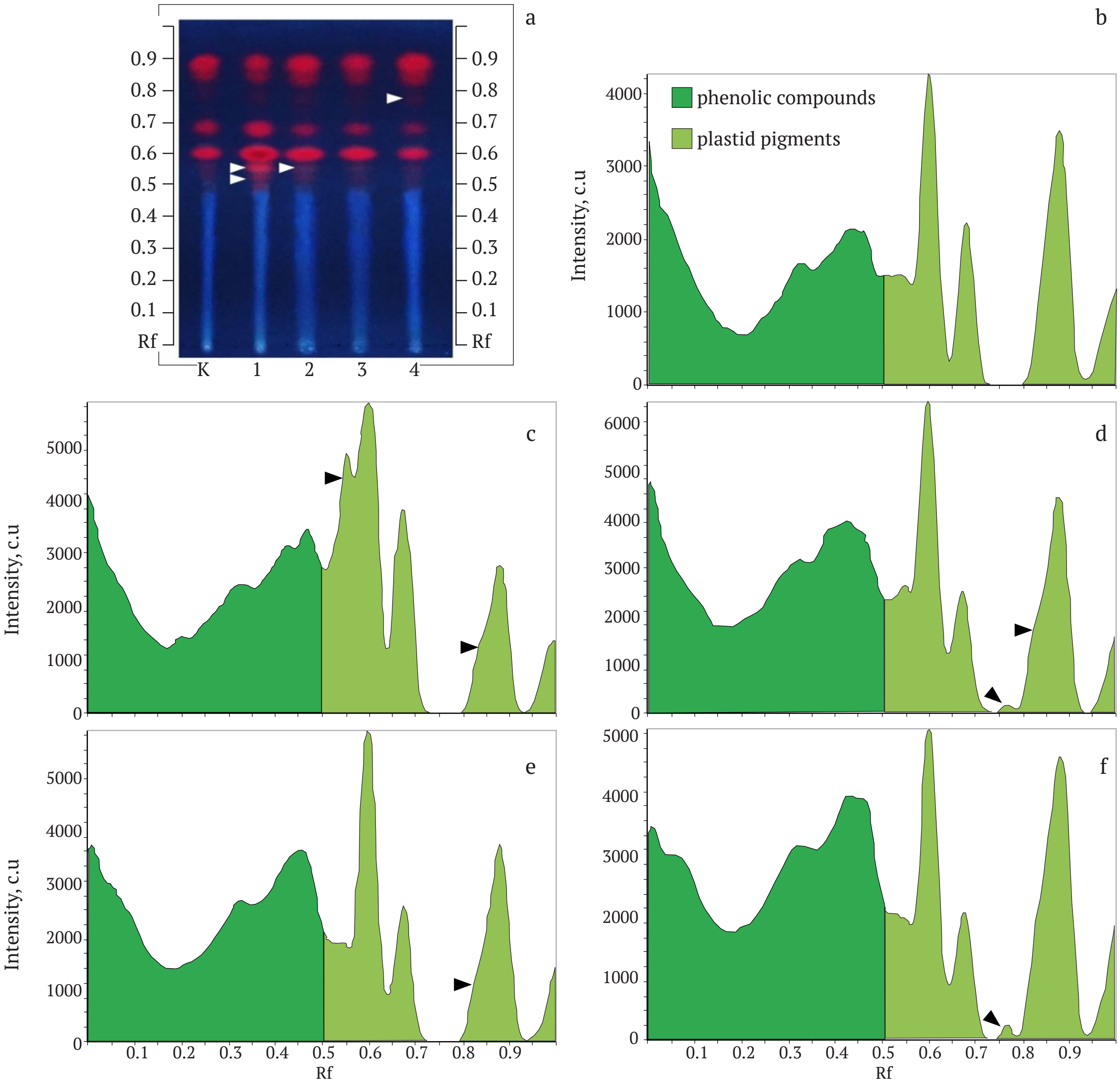

Figure 4. Photodensitograms of plastid pigments in cotyledons of Scots pine seedlings on Day 14 of germination; *b* – control; *c*, *d*, *e*, *f* – photodensitograms of seedling extracts that were pretreated with solutions with concentrations of 10, 15, 20, and 40 µg/ml, respectively; arrows show peaks that differ significantly in their area from the control group

A different trend was observed in the qualitative composition of phenolic compounds. Under conditions of increasing the concentration of the phenol-ammonium complex, the number of phenols in seedling tissues increased (Fig. 5).

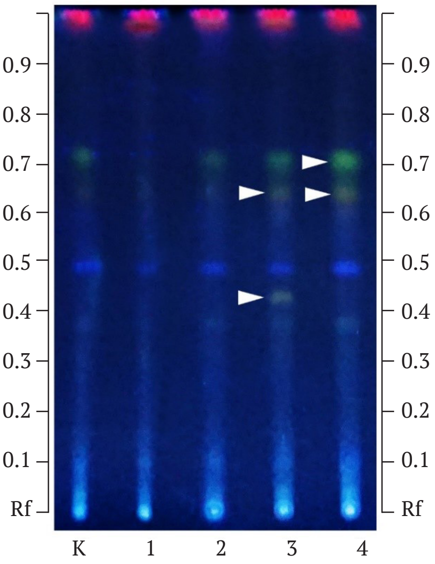

Figure 5. Chromatogram of phenolic compounds of Scots pine seedlings on Day 14 of germination; *K* – control, *1-4* – plant extracts after seed treatment with rutin-ammonium complex with concentrations of 10, 15, 20, and 40 µg/ml, respectively; arrows show flavonoids, the content of which is much higher than the control

When seeds were treated with a solution with a concentration of 20 and 40 µg/ml in methanol extracts, the content of flavonoids with Rf ~0.63 and 0.71 significantly increased (Fig. 5, 3, 4). The compound with an Rf of ~0.43 was found exclusively in seedling extracts treated with a solution of 20 µg/ml. Treatment of seeds with rutin-ammonium complex with the lowest concentration caused the opposite effect. Under such conditions, the qualitative composition and total pool of phenolic compounds in seedling tissues decreased (Fig. 5, 1).

The opposite dependence of the synthesis of phenols and terpenoids can be caused by different ways of synthesis of these compounds, except for flavonoids, the development of which can compete with carotenoids for a substrate common to the corresponding enzymes. Carotenoids are polyene derivatives of isoprene, whose molecules have double bonds. These pigments are mainly localised in chloroplasts, where they are synthesised from acetyl-CoA (Della Penna and Pogson, 2006). Most plant phenols are formed from shikimic acid in chloroplasts and cytosols. At the same time, the synthesis of flavonoids and carotenoids is also possible from acetyl-CoA under conditions of its carboxylation in acetyl-CoA carboxylase and the subsequent establishment of malonyl-CoA, which is a substrate for the synthesis of flavonoids. Therefore, the accumulation of flavonoids in seedlings against the background of a simultaneous decrease in the content of carotenes and xanthophylls may indicate the ability of the phenol-ammonium complex to selectively affect the enzyme systems responsible for the synthesis of substances formed from acetyl-CoA.

Conclusions

Quercetin glycoside rutin (quercetin-3-o-rutinoside) after interaction with an aqueous solution of 10% ammonia forms a complex of medium- and high-polar organic compounds with high biological activity.

These substances, when treated with loblolly pine seeds, can affect germination energy, germinating capacity, and subsequently seedling growth. The effect of the complex of organic compounds formed from the rutin-ammonium complex depends on the concentration and duration of seed treatment.

A solution with a concentration of 15 µg/ml increased seed germination, while concentrations of 10 and 15 µg/ml promoted the synthesis of plastid pigments. The total pool of phenolic compounds increases when seeds are treated with solutions with concentrations of 20 and 40 µg/ml. Therefore, the phenol-ammonium complex is able to regulate the processes of growth and development, and affect the secondary metabolism of Scots pine seedlings. The identified effects reveal a new, still poorly understood aspect of the role of flavonoids in the plant body.

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Вплив рутин-амонійного комплексу на фізіологічний стан проростків сосни звичайної

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Анотація. У рослинному організмі фенольні сполуки неспецифічно впливають на процеси морфогенезу і виконують широкий спектр регуляторних і захисних функцій. Особливий інтерес становлять процеси, які пов'язані з комплексотворенням флавоноїдів у результаті їхньої взаємодії з амонійними формами азоту. Полярні сполуки, які утворюються в тканинах у результаті хімічної трансформації, достатньо рухомі у ґрунтових розчинах і виявляють високу біологічну активність. Властивості фенол-амонійних комплексів викликають значне зацікавлення в аспекті морфогенезу, фізіології стійкості, а також у системі взаємодії рослин із ґрунтовими мікроорганізмами. Це також надзвичайно актуально при виробництві якісного та стійкого до несприятливих факторів садивного матеріалу. Дослідження впливу фенол-амонійного комплексу проводили на насінні і проростках сосни звичайної. Кількісні показники енергії проростання та схожості визначали методом пророщування насіння. Біохімічне профілювання екстрактів тканин проростків виконували методом високоефективної тонкошарової хроматографії. Експериментально підтверджено, що рутин (кверцетин-3-О-рутинозид) після взаємодії з 10 % водним розчином аміаку утворює комплекс речовин, серед яких методом хроматографії виявлено полярні продукти, які потенційно впливають на регуляцію росту. За сумарної концентрації 15 мг/л ці речовини достовірно підвищували показники енергії проростання та схожості насіння. У проростків сосни вони стимулювали ріст коренів і пагонів. Ефект дії комплексу органічних сполук на проростки залежав від концентрації, тривалості оброблення насіння і мав пролонговану дію. Отриманий фенол-амонійний комплекс за концентрацією 10-15 мг/л сприяв підвищенню кількості хлорофілів, каротиноїдів у тканинах проростків, а за 20-40 мг/л збільшував уміст продуктів фенольного синтезу

Ключові слова: рутин, флавоноїди, амоній, регулятор росту, сосна звичайна, насіння