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Effect of Phytohormones of Kinetin and Epibrassinolide on Content and Intracellular Localization of Glucosides and Free Amino Acids in Pea Plants Cells (*Pisum sativum L.*)

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Abstract: During injection of kinetin and epibrassinolide in pea seedlings (*Pisum sativum L.*) an increase of content of specific for this compound isosuccinimide-β-glucoside (IS-glucoside) for 15-30%, ethyl-β-glucoside for 40-80%, GABA free amino acids, glutamate and alanine only under kinetin for 15-20% was observed. The field experiments showed an increase of aglycone content but the amount of IS-glucoside decreased at first and then increased 14 days after plant treatment with phytohormones. It was shown that effect of phytohormones on content of IS-glucosides and free amino acids was dependent on action time and age of plant. By using membrane trophic compound of DMSO it was discovered that in pea seedlings cells the vacuolar fund of IS-glucoside was 70% of initial and aglycone with γ-aminobutyric acid as its precursor were localized in cytoplasm. Aspartate and glutamate were almost equally distributed between cytoplasmic and vacuolar cell funds. It is suggested that reactions of pyrrolidone structure of IS-glucoside aglycone formation and synthesis of the glucoside were processed in cytoplasm. Further IS-glucoside was transferred and stored in vacuoles of pea seedlings cells by contrast with ethyl-β-glucoside mainly localized in cytoplasm.

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1. INTRODUCTION

Along with proteins, carbohydrates, lipids and vitamins in plants there are the substances usually so-called of secondary metabolites. One of the most important groups of secondary metabolites are the glycosides. In natural glycosides an O-glycosidic bond connects residuals of monosaccharides with non-hydrocarbon components (aglycones). Relative ease of formation and cleavage of all types' glycosidic bonds provides metabolic versatility of relevant compounds in living cell and explains wide occurrence of this structural unit in living systems [1]. Glycosides perform different roles in plants including transport, protective [1] and detoxicative such as transformation of toxic ethanol accumulated under hypoxia into inert compound of ethyl-β-glucoside [3]. Chemical nature of glucosidic donor is limited to relatively small group of compounds. Most frequently the content of glucosides has glucose. The nature

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of acceptor is diverse. The environment in the form of glucosides possesses alcohol and phenols which have mainly β-configuration but also saponins and alkaloids. Therefore, the process of phenol compounds glucosidation is an exclusive characteristic of higher plants [4]. Various types of glucosides are mainly localized in vacuolar compartment of plant cells [5].

Discovered that pea plants (*Pisum sativum L.*) have a specific glucoside which was extracted, purified and identified as 5-oxy-2-pyrrolidone-β-glucopyranoside and matched with isosuccinimide-β-glucoside (IS-glucoside) also found in this plant per UV- and IR-spectrophotometry, melting temperature and hydrolysates [5,6]. Using different C^{14} -amino acids we determined that aglycone of IS-glucoside is a cyclic derivative of nonproteinogenic γ-aminobutyric acid [7]. The significant fluctuation of IS-glucoside content was dependent on the kind of pea plants and ontogenesis stage [8]. Found that IS-glucoside is generated during seed germination and plays and important role in young, growing organs and tissues. Maximum amounts of this glucoside present in green parts of seedlings. Etiolated plants contain its' smaller quantities as well as aglycone [8].

It is well known that origin of all ontogenesis stages is under hormone control and a possibility of regulation by exogenous plant growth regulators of special exchange processes in plant cells is also shown for various classes of secondary metabolites [2]. An effect phytohormones of cytokinin and epibrassinolide (EBL) have on IS-glucoside content and related to its metabolism compounds including amino acids was investigated. The volumes of some intracellular funds of these compounds were analyzed.

2. MATERIAL AND METHODS

The experiments were conducted with 10-15 days old pea seedlings "Ramonskiy 77" grown in laboratory conditions by hydroponics method under 12h photoperiod. The plant material was obtained from the Department of Plant Selection and Seed Breeding of the Voronezh State Agrarian University (Russia). In some experiments the plants in bud formation stage grown under field conditions were used. In all experiments performed the plants were subjected to treatment with phytohormone solutions of cytokinin and EBL of 10mg/l concentration. Under laboratory conditions the phytohormone solutions were injected by transpiration stream into over ground part of seedlings during 12-24hr. In the field experiments the plants were sprayed with phytohormone solutions and the leaves were analyzed after 7-14 days. The content of IS-glucoside and its aglycone in plant cells was determined by our method after chromatographic separation of uncharged compound by absorption spectrophotometry of 208nm and 212nm [7]. Amount of ethyl-β-glucoside was calculated after hydrolysis by using glucooxidase method per generated glucose [8]. Content of certain amino acids was determined after ninhydrin reaction [9].

Isolation of cytoplasmatic and vacuolar cell funds was investigated using membrane trophic compound of dimethyl sulfoxide (DMSO) previously used for cell funds analysis [6]. During preliminary experiments, we found the DMSO concentrations which can selectively change permeability of only plasmalemma or tonoplast in pea seedlings leaf cells. Disruption of tonoplast permeability was estimated by neutral red output used for treatment of hewn leaves for 20-180 min. Output of colorant from cells was calculated according to change of concentration under 530 nm and damage level of tonoplast was controlled by microscopic display. The experiments showed that disruption of plasmalemma happens under DMSO concentration of 7.5-15.0% and of tonoplast – around 50%. To analyze an intracellular localization of glucosides and amino acids a quantity of leaves (0.5g) was shred and placed into DMSO solution of matched concentrations. The yield of analyzed compounds was studied through the analysis of solutions after chromatographic separation on plates with silica gel G and calculated on mg of protein.

All experiments were conducted in 3 biological and 2 chemical replications and were processed by mathematical statistics methods with $p>0.05$. The presented data is the arithmetical mean plus the error. Data of one of the typical experiments is presented in current paper.

3. RESULTS AND DISCUSSION

We investigated an impact of kinetin and EBL on content of IS-glucoside and its aglycone and on ethyl-β-glucoside, glucose and free amino acids - GABA, glutamate, alanine and aspartate. The data shown (Figure 1. and Figure 2) suggests that growth regulators influenced the content of investigated compounds to different extent. It was discovered that in 2-week-old seedlings kinetin and EBL after 12hr of injection stimulated reactions of IS-glucoside synthesis followed by increase of IS-glucoside for 30% and 11% respectively. At the same time the content of aglycone and endogenous glucose in plant cells was decreasing. At the same time the level of free amino acids – GABA, glutamate, aspartate and alanine – was increasing. But after 24hr of injection both kinetin and EBL were decreasing the content of GABA and glutamate. With that the content of IS-glucoside and glucose was also decreasing in the presence of high speed of aglycone and ethyl-β-glucoside formation which suggests about the speed change of these compounds synthesis under various duration term of phytohormones on pea seedlings.

When the plants were treated with kinetin on latter ontogenesis stages i.e. during flower bud formation then 7 days after the aglycone content in pea seedlings leaves was increasing for 20% relative to control and GABA for 28% respectively. In the meantime, the synthesis of IS-glucoside was suppressed since its content was decreased for 40%. The decrease of free amino acids content in average for 7-8% was noted. Our data shows (Figure 1.) that kinetin did not have an impact on synthesis of ethyl-β-glucoside and glucose content. In 14 days after the treatment the content of aglycone increased 1.5-fold while the GABA content did not change.

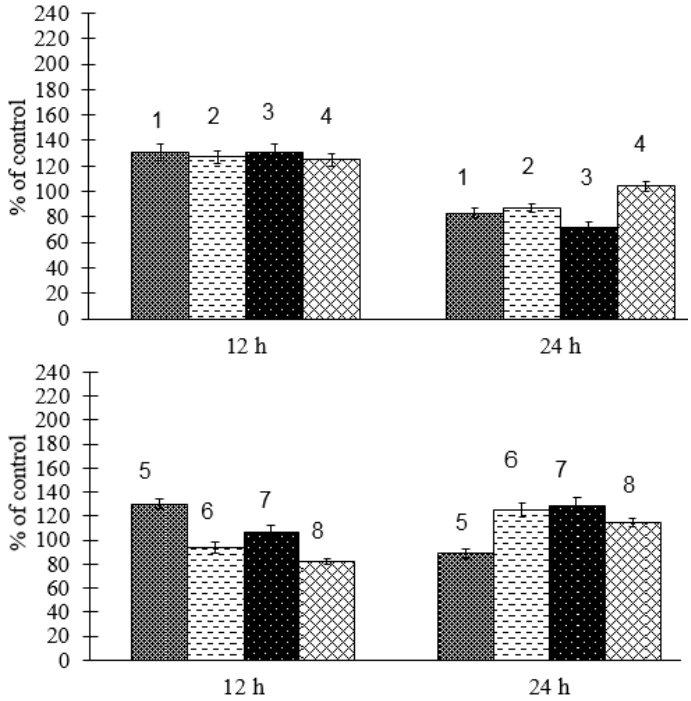

Figure 1. Impact of kinetin on content of amino acids and IS -glucoside in pea plants under 12 and 24h of injection: 1 – aspartate, 2 – glutamate, 3 – alanine, 4 – GABA, 5 – IS-glucoside, 6 – aglycone, 7 – ethyl-β-glucoside, 8 – glucose

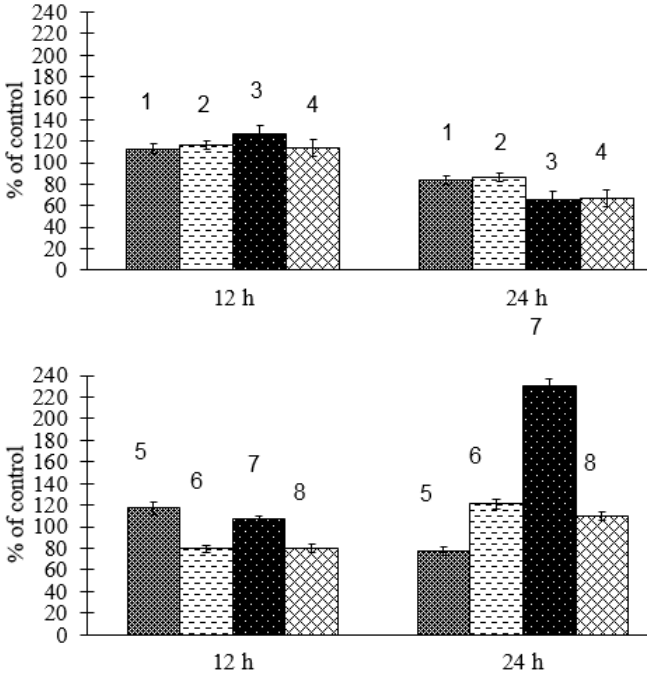

Figure 2. Effect of EBL on content of amino acids and IS-glucoside in pea plants under 12 and 24h of injection: 1 – aspartate, 2 – glutamate, 3 – alanine, 4 – GABA, 5 – IS-glucoside, 6 – aglycone, 7 – ethyl-β-glucoside, 8 - glucose

It can be assumed that the pea plants have relatively active enzyme systems participating in cyclization of GABA with the formation of pyrrolidone structure of IS-glucoside aglycone as showed before [7] and systems able to synthetize the glucoside itself. This was evidenced by increase on 20% of the aglycone content in pea plants cells after 14 days since treatment compare to the control plants. Beyond that kinetin contributed to glucose accumulation. The phytohormone stimulated also alanine formation but decreased the amounts of aspartate and glutamate.

Special aspect of cell structure is a complexity of its dimensional organization. Specificity in demonstration of metabolic qualities of the same compounds is a result of their spatial isolation i.e. compartmentation. In plant cells there are reserve vacuolar and active cytoplasmic funds [10]. Diverse compartmentation of compounds and enzymes of their metabolism determines speed of exchange processes of cells. Vacuole plays an important role in storage of not only primary but also secondary metabolites [5,10]. However vacuolar funds of low molecular compounds such as amino acids and glucosides are investigated only for a small number of plants. For this reason, further, we analyzed the allocation of isosuccinimide-β-glucoside, its aglycone and several free amino acids related to their metabolism between cytoplasmic and vacuolar cell funds of pea seedlings. Extraction of the funds was conducted by method using a membrane trophic compound DMSO.

The data presented in Table 1 and Table 2 show results of studying compounds distribution between vacuolar and cytoplasmic cell funds of pea seedlings. In Table 1 we can see that most quantity of IS-glucoside, up to 70% of total cell content, is accumulated in vacuole. As it was discovered [1] most of plant glucosides have vacuolar localization. The vacuole being a multifunctional organelle can reserve relatively large number of compounds including glucosides thus isolating from enzymes of their catabolism – β-glucosidases. Such spatial designation allows an itself existence of glucosides in plant cells and perform functions of defense and detoxication. Therefore, an IS-glucoside aglycone was mainly localized in

cytoplasm, where 83% of total cell fund of this compound was accumulated as it was suggested by the experiment results.

Table 1. Content of IS-glucoside and aglycone in vacuolar compartment and cytoplasm (a – μmol/gr of fresh weight, b - % of total cell content)

Compounds	cell		cytoplasm		vacuole	
	a	b	a	b	a	b
IS-glucoside	3.00±0.10	100	0.96±0.04	32	2.04±0.08	68
Aglycone	4.10±0.20	100	3.41±0.08	83	0.70±0.02	17
Ethyl-β-glucoside	0.26±0.005	100	0.16±0.1	61	0.10±0.005	39

It was discovered that primary spot of GABA formation in cells is cytoplasm [9]. At the same time, it was shown [11] that vacuolar fund can contain significant amounts of this amino acid. However, such researches are occasional. As shown in Table 2, we analyzed allocation of GABA and several free amino acids between cytoplasmic and vacuolar cell funds of pea seedlings using membrane trophic DMSO compound.

Table 2. Intracellular allocation of amino acids in pea seedlings (a – μmol/gr of fresh weight, b - % of total cell content)

Compounds	cell		cytosol		vacuole	
	a	b	a	b	a	b
GABA	3.93±0.05	100	2.57±0.10	65	1.30±0.02	33
Glutamate	6.72±0.31	100	3.56±0.16	53	3.00±0.13	46
Alanine	4.04±0.20	100	2.91±0.09	72	1.13±0.04	26
Aspartate	8.57±0.13	100	4.30±0.10	50	4.28±0.15	50

The results showed that GABA and alanine had preferential cytoplasmic localization with the content of 65% and 72% of total cell pool respectively. With that the contents of aspartate and glutamate were approximately equally distributed between cytoplasmic and vacuolar cell funds. Obtained results are corresponded to other works [9] suggest that alanine, glutamate and GABA in cells of some plants are concentrated substantially in cytoplasmic cell funds.

It was shown before that GABA in synthetized in cytoplasm where the enzyme of glutamic acid decarboxylase (GAD) is localized. In that context, it can be assumed that reactions of cyclization of carbon skeleton of GABA accompanied by pyrrolidone structure formation of IS-glucoside are particularly processed in cytoplasm. Probably the reactions of IS-glucoside formation are also processed in cytoplasm [7]. At the same time the generated glucoside later could be stored in vacuolar fund of pea seedlings cells which is suggested by our results.

4. CONCLUSION

Our research showed that growth regulators such as kinetin and epibrassinolide have an impact on content of isosuccinimide-β-glucoside - a pea seedlings specific glucoside. Interconnection of content change for glucoside, its aglycone, free glucose and GABA in pea seedlings under the influence of phytohormones was noted. Therefore, the phytohormones' effect on content of studied glucoside and aglycone was determined as by activity period of phytohormones as by age of the treated plants. Mechanism of change of IS-glucoside

biosynthesis under influence of growth regulators can be discussed. Capability of phytohormones to affect the activity of the number of enzymes and increase permeability of membrane structure which contribute to better conditions of intracellular metabolite transport were shown [12]. In this case stimulation of glucoside formation after plant treatment with kinetin can be considered as an outcome of significant improvement of supply of enzyme biosynthesis by substrates due to reduction of membrane barriers preventing entry of precursor molecules to the points of use.

While investigating intracellular localization of IS-glucoside, its aglycone and number of free amino acids related to their exchange while using membrane trophic DMSO compound it was discovered that in pea seedlings cells there are at least two IS-glucoside funds – cytoplasmic and vacuolar ones. In this case, vacuolar fund of IS-glucoside was about 70% of its cell pool. At the same time aglycone and its precursor – γ-amino butyric acid- were localized mainly in cytoplasm. This proves the hypothesis [7] that the reactions processed right in the cytoplasm were: formation of pyrrolidone structure of aglycone and synthesis of the glucoside itself further to be transferred to and stored in vacuoles of seedlings cells. The vacuole can reserve relatively large number of glucosides thus isolating from enzymes of their catabolism – β-glucosidases. Such spatial designation allows an itself existence of glucosides in plant cells and perform functions of defense and detoxication.

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