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Impact of Salicylic Acid on Symbiotic Relations Between Peas and *Rhizobium leguminosarum* bv. *viceae*

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Abstract: The study was targeted at the investigation of exogenous salicylic acid (SA) impact on bacteria proliferation *in vitro,* rhizobia penetration in the root tissues, the SA and hydrogen peroxide (H_2O_2) content in the root seedlings under inoculation of pea by compatible strain *Rhizobium leguminosarum* bv. *viceae.* Depending on the concentration SA either did not affect (0.0036-0.014 mM) rhizobia proliferation or suppressed their growth (0.07-0.2 mM) *in vitro*. Exogenous SA (0.2 mM) inhibited rhizobia penetration in the root tissues (by 2 and 5 times depending on pH medium) and contributed to the increase of endogenous SA and H_2O_2 content in the tissues. Various possible mechanisms of SA impact on rhizobial symbiosis are discussed.

Key words: *Pisum sativum* L., *Rhizobium leguminosarum* bv. *viceae,* root bacteria proliferation, salicylic acid, hydrogen peroxide

INTRODUCTION

Salicylic acid (SA) is synthesized by many plants[1] and is known as one of the possible signal molecules participating in the reactions of hypersensitivity cells and formation of systemic acquired resistance (SAR) of the plants to bacterial, fungi and viral infections[2-5]. SA also participates in thermogenesis in the plants[1] and accumulates in the plant tissues under the impact of unfavorable abiotic factors, contributing, in particular, to the increase of plants resistance to high and low temperatures, salinization[6,7].

In the recent works the role of SA in symbiotrophic plant organisms is discussed. Thus, according to Martinez-Abarca *et al.*[8] SA accumulates in alfalfa roots in case of incompatible species symbiosis. The same phenomenon is observed in peas during plants inoculation by rhizobial strain deficient in Nod-factor synthesis[9]. Exogenous SA induced in legumes roots inhibited nodules primordial formation[8] and reduced the number of nodules on legumes roots[10]. According to Garcia-Garrido and Ocampo[11] in symbiotrophic organisms (arbuscular-mycorhisa and rhizobial types) SA may be involved in the regulation of protective reactions of the host plant.

Nevertheless, literary data confirming SA participation in the interactions of symbiotrophic organisms are extremely scarce. This made the authors of the present work to pursue and objective to reveal a number of phenomenological SA impacts on symbiotic partners peas and compatible strain *Rhizobium leguminosarum* bv. *viceae* (bacteria proliferation *in vitro,* their penetration in the root tissues, content of endogenous SA and H_2O_2).

MATERIALS AND METHODS

Pea seeds (*Pisum sativum* L.) of Marat variety before sprouting were sterilized on the surface by 3% solution of hydrogen peroxide for 15 min with further thorough washing by distilled water. The seeds were put on wet filter paper in the vats and sprouted at 22°C during two days. Then root seedling of identical size were selected and inoculated by the suspension of cells of *Rhizobium leguminosarum* bv. *viceae* (strain 245) in the concentration $2x10^8$ cells ml^{-1} based on 1 ml $root^{-1}$ (strain was received from the Institute of Leguminous and Groats Cultivars of the Russian Academy of Agricultural Sciences, Russia).

While studying SA impact on symbiotic partners SA solution of certain concentration was added to the vats instead of water. SA was first dissolved in a small amount of 96% ethanol. Sprouts incubation medium was neutralized by 0.1 N solution of NaOH. Inoculated pea seedlings in the vats were placed in thermostat at 22°C and left for 2 days. Then seedlings were used for analytical and other purposes.

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Rhizobium leguminosarum bv. *viceae* growing in the cultivar was first conducted in solid, then in liquid medium as per method[12]. The impact of SA in different concentration on rhizobia proliferation was assessed by the change of optical density of the bacteria suspense ($À_{590}$) with the help of ФЭК КФК-2 (Zagorsk optical-mechanical works, Russia). The measurements were conducted against media without bacteria and the SA impact degree on the bacteria growth was expressed in percent of control (medium with bacteria but without SA).

In the course of studying of SA impact on rhizobia penetration in the root tissues upon inoculation termination the roots were subjected to washing from bacteria adsorbed on their surface by phosphate buffer (pH 7.4) within 15 min using a shaker (Elpan, type 358 S, Poland). Then the roots were homogenized in distilled water. Bacteria inoculation from the suspense acquired was carried out on the solid agar medium with 0.05 ml petridish^{-1}. Dilution is 1:1000. The intensity of bacteria penetration in the root was assessed by the number of sprouting colonies. One colony corresponded to one penetrating bacterium forming this very colony.

Prior to SA content determination the roots were thoroughly washed by distilled water and then fixed by 96% ethanol. 3-4 fold extraction of phenolic compounds from 1.0-1.5 g of homogenized roots (homogenate was obtained in the presence of quartz sand and 80% ethanol) was carried out in bain-marie at 80-90°C. The volume of ethanol extraction was measured and split into two parts to determine free SA content in one of them and associated SA content in the other. Further extraction of phenolic compounds was conducted as per the scheme[13] which allows to separate from the total mass the groups of "acid phenolic compounds" including SA and other phenolic carbon acids. Ethanol solutions acquired as a result of extraction as per the scheme identified containing free and acquired as a result of acid hydrolysis phenolic compounds were chromatographed in the thin layer on the plates of 150x150 mm, with silufol applied (Silufol UV 254, Kavalier, Czechoslovakia). Free phenolic compounds were separated in one direction in the system: toluol-dioxane-acetic acid (90:25:4 by volume). The compounds obtained after hydrolysis (containing SA conjugates) were additionally split in the other direction in the system: chloroform-ethanol (4:1 by volume). Chromatograms were analyzed in ultraviolet rays. SA location was determined by the location of authentic SA sample applied either on one and the same plate with the extractions for the cases of separation in one direction, or on a separate plate with the division in two directions. SA determination was carried out spectrophotometrically with the help of SF-56Ì (LOMO, Russia) equipped by computer to register absorption at wave length 300 nm. Commercial SA preparation (Russia) was used for the tests and construction of the calibration curve. The content of associated SA was calculated by the difference in extractions subjected and not subjected to acid hydrolysis.

Hydrogen peroxide content in the roots was determined by Ugarova *et al.*[14].

RESULTS AND DISCUSSION

Figure 1 presents the data of the test aimed at the study of exogenous SA impact on the cultivar growth *Rhizobium leguminosarum* bv. *viceae in vitro*. At concentrations from 0.5 to 2.0 μg ml^{-1} (0.0036-0.014 mM) there was no SA impact on bacteria growth. Significant inhibition of bacteria growth was observed at SA concentration 10 μg ml^{-1} (0.07 mM): approximately by 20% at both expositions. SA concentration 28 μg ml^{-1} (0.2 mM) made a considerable inhibiting impact on rhizobia growth: by 50% with the exposition of 16 h and by 80% with the exposition of 24 h.

Thus, in high concentration SA produced negative impact on rhizobia growth *in vitro*. Its negative influence on rhizobia may show already in rhizosphere and roots surface. It can also be assumed that rhizobia penetrating into the root tissues may be subject to negative impact of plant SA in the course of proliferation.

According to the data of Martinez-Abarca *et al.*[8] SA exogenously induced in alfalfa roots at the concentration of 25 μM inhibited nodules primordia formation by 75%. This allows the authors to assume that SA may participate in the nodule organogenesis. It would be logical to suppose that SA may affect the processes associated with rhizobia penetration into the root tissues. In the tests performed pea roots inoculation was carried out in the presence of exogenous SA (0.2 mM) for 48 h. According to the Table 1, exogenous SA made a negative impact on rhizobia penetration in the root tissues reducing the number of sprouting bacteria colonies almost by half at medium pH of 4.0 and by 5 times at neutral pH.

More intense inhibition of rhizobia penetration in the roots at neutral medium reaction may apparently be accounted for by easier roots absorption of sodium salicylate produced in the course of SA alkaline neutralization and consequently by more pronounced SA negative impact on rhizobia. This is confirmed by the data on determination of endogenous SA content in the roots (Table 2). There might be another explanation connected with pH impact on the start-up of plant symbiotic or protective reactions in response to rhizobial infection[15].

Table 1: Exogenous salicylic acid impact (0.2 mM) on the number of sprouting of *Rhizobium leguminosarum* extracted from the pea root tissues

Variant	Number of sprouting bacterial colonies
Control (inoculation, medium pH 6.5)	36.5±4.06
Inoculation+CK (medium pH 4.0)	16.3±3.18
Inoculation+CK (medium pH 6.45)	7.3±2.80

Table 2: Salicylic acid content in the pea roots subjected to various factors

	SA content μg g^{-1} of fresh root mass	
Variant	Free SA	Conjugated SA
Control (H_2O)	0.63±0.12	8.81±1.67
Inoculation for 2 days, pH 6.5	3.73±0.64	9.62±1.66
Inoculation+SA (0.2 mM), pH 4.0	5.21±0.71	8.08±1.10
Inoculation+SA (0.2 mM), pH 6.45	8.40±1.16	11.00±1.83
H_2O+SA (0.2 mM), pH 4.0	14.05±2.36	27.69±4.64

Table 3: Hydrogen peroxide content in the pea roots subjected to salicylic acid action

Variant	H_2O_2 content μg g^{-1} of fresh roots mass
Control (H_2O)	3.50±0.29
Inoculation for 2 days, pH 6.5	1.82±0.17
Inoculation+SA (0.2 mM), pH 4.0	6.91±0.59
Inoculation+SA (0.2 mM), pH 6.45	10.29±1.01
H_2O+SA (0.2 mM), pH 4.0	13.75±1.10

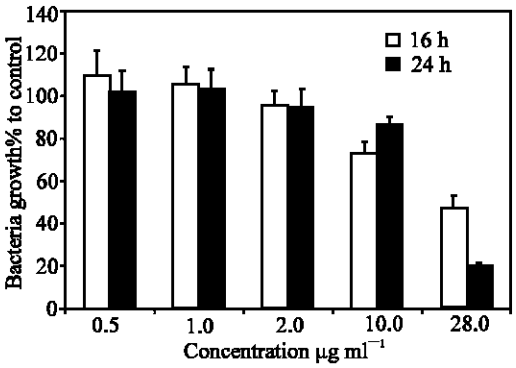

Fig. 1: Salicylic acid impact on *Rhizobium leguminosarum* bv. *viciae* proliferation 16, 24 h-exposition duration as of introducing salicylic acid in the bacteria growth medium in the concentrations stated

It follows from Table 2 that depending on such factors as inoculation, content of exogenous SA in the medium and pH, the amount of endogenous SA changes significantly. Inoculation contributes to 6-fold increase of free SA content. Similar facts were observed in the tests by Blilou *et al.*[9] with pea roots 48 and 72 h upon inoculation by their compatible strain *Rhizobium leguminosarum*.

This allows to presume that at initial stage of the infection process there are started up some mechanisms contributing to SA synthesis by macrosymbionts. This may be connected with autoregulation of initial symbiosis stages aimed in particular at quantitative regulation of rhizobia penetration in root tissues. This is confirmed by data acquired by Akimova *et al.*[16] which speak in favor of the fact that individual parts of pea roots seedlings possess different sensitivity to rhizobia penetration. This is accompanied by different activity (along the root length) of peroxidase, which according to the authors quoted and others[17] play a protective role in rhizobial symbiosis, in particular, by intensification of cell walls lignification which prevents bacteria penetration in the roots. According to Vasilieva *et al.*[18] one day after inoculation of pea roots seedlings by incompatible strain *Rhizobium leguminosarum* bv. *phaseoli in* the roots there intensified generation of super-oxide radical O_2^-.

Therefore, regulation by leguma of rhizobia penetration in root tissues may be associated with the activation of protective systems in macrosymbiont, as well as in the case of pathogen penetration in the plant. According to Leon *et al.*[19] pathogen infection results in the start-up of protective reaction in tobacco leaves, which is accompanied by intensification of generation of free oxygen radicals including hydrogen peroxide, which in its turn activates hydrolase of benzoic acid catalyzing SA synthesis.

In our tests this is confirmed by the increase of endogenous SA and H_2O_2 content in the roots in the course of rhizobia inoculation with the addition of exogenous SA (Table 2 and 3). Exogenous SA to a large extent increased free SA content in the roots: by 140% at pH 4.0 and by 225% at pH 6.45. The content of associated SA did not increase reliably. SA accumulation was accompanied by intense H_2O_2 formation at neutral medium reaction to a larger extent than at acidic reaction (Table 3).

The link between SA accumulation and the increase of hydrogen peroxide content in the tissues may be accounted for by literary data. H_2O_2 accumulation in plant tissues is known to happen not only at the expense of its formation in different reactions, but also by inhibiting enzymes catalyzing its dissociation-catalase and peroxidase[20]. One of such catalase inhibitors contributing to hydrogen peroxide accumulation is SA which in competing inhibiting is associated with active catalase center[3]. At the same time, as mentioned above the increase of H_2O_2 level in its turn induces SA accumulation and its immediate precursor-benzoic acid, as well as fast activation of 2-hydroxilase of benzoic acid catalyzing SA formation from benzoic acid[19]. H_2O_2 accumulation in arabidopsis leaves when treating the plants by exogenous SA was shown by Rao *et al.*[21].

Notable are the data in the option with roots treatment by exogenous SA without seedlings inoculation by rhizobia (pH 4.0) (Table 2 and 3). In this variant the content of endogenous SA and H_2O_2 increases to a large

extent as compared to analogous variants with inoculation (by 2.0-2.7 times). This, apparently, speaks in favour of rhizobia impact on the processes of SA and H_2O_2 accumulation. This influence is presumably mediated via rhizobial Nod-factor suppressing protective system of the host plant[11]. According to Schulze and Kondorosi[22] rhizobia produce unidentified elicitor, which, in association with the plant receptor, induces SA accumulation and initiates the start-up of protective reactions. Rhizobial Nod-factor (lipohitooligosacharide), compatible with the plant receptor, prevents SA synthesis and blocks protective reactions. Besides, it should be noted that in the option with roots treatment by exogenous SA (without inoculation) the content of associated SA increases considerably (Table 2). This is likely to prove intensification of the processes of conjugation of free SA with the formation of glucosides and glucosides ethers[5], which are biologically less active than free phenolcarbon acids SA included CK[23]. inoculation obviously tells on the SA association processes.

Therefore, the data drawn allow to conclude that SA may be involved in the establishment of symbiotic relationships between peas and rhizobia. Its role may be connected with the protective reactions of the host plant and be revealed with incompatible symbiosis, macrosymbiont regulation of infection and nodulation processes (with compatible symbiosis) and with the action of unfavorable abiotic factors making a negative impact on symbiosis.

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