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SEARCH FOR ECOLOGICAL METHODS TO ENLARGE SYMBIOTIC NITROGEN FIXATION EFFICIENCY BY PEA (*Pisum sativum* L.)

Summary

Conducted till now studies on symbiosis of papilionaceous plants and rhizobia discovered the genetic background of this phenomenon and allowed to identify numerous plant and bacteria metabolites involved in the process of starting of symbiosis and development of nodules. One of this compounds are flavonoids, recognised as signal particles participating in information exchange between a bacteria and a plant, affecting among others nodules formation on papilionaceous plants. These compounds are often submitted to processes which decrease their concentration on the way from a producer to a destined organism, they diffuse or break-up in the soil solution and are degraded by soil microorganisms. It can be then assumed that their insufficient amount is a cause of low efficient symbiosis, what considerably limits plant supply with nitrogen and decreases their yielding. An effect of use of flavonoid extract obtained from germinating seeds on ontogenesis, forming of physiological growth indexes and yielding of pea was evaluated in these studies. Application of a flavonoid preparation increased, among others, the number and weight of nodules and activity of nitrogenase, what in a consequence led to production of greater mass of vegetative and generative organs by pea plants.
Key words: *pea, flavonoid preparation, seed dressing, nitrogenase, nodule, development of plant*

POSZUKIWANIE EKOLOGICZNYCH METOD ZWIĘKSZENIA WYDAJNOŚCI SYMBIOTYCZNEGO WIĄZANIA AZOTU PRZEZ GROCH SIEWNY (*Pisum sativum* L.)

Streszczenie

Prowadzone dotychczas badania nad symbiozą roślin motylkowatych i rizobiów odkryły podłoże genetyczne tego zjawiska i pozwoliły na zidentyfikowanie licznych metabolitów roślinnych i bakteryjnych zaangażowanych w proces nawiązania symbiozy i rozwój brodawek korzeniowych. Jednym z takich związków są flawonoidy, uznawane za cząstki sygnalne uczestniczące w wymianie informacji pomiędzy bakterią i rośliną, wpływające między innymi na powstawanie brodawek korzeniowych na roślinach motylkowatych. Związki te często podlegają procesom powodującym zmniejszenie ich stężenia na drodze od producenta do organizmu docelowego, dyfundują lub rozpadają się w roztworze glebowym oraz są degradowane przez mikroorganizmy glebowe. Można zatem przypuszczać, że niedostateczna ich ilość jest przyczyną mało wydajnej symbiozy, co znacznie ogranicza zaopatrzenie roślin w azot i zmniejsza ich plonowanie. W badaniach określano wpływ stosowania wyciągu flawonoidowego z kiełkujących nasion na ontogenezę, kształtowanie fizjologicznych wskaźników wzrostu oraz plonowanie grochu siewnego. Zastosowanie preparatu flawonoidowego zwiększyło między innymi liczbę i masę brodawek korzeniowych oraz aktywność nitrogenazy, co w konsekwencji prowadziło do wytwarzania większej masy organów wegetatywnych i generatywnych przez rośliny grochu siewnego.
Słowa kluczowe*: groch siewny, preparat flawonoidowy, moczenie nasion, nitrogenaza, brodawki korzeniowe, rozwój roślin*

1. Introduction

Pea is an important species among legumes cultivated in our climatic zone [19, 25]. One of the main cause of its small cultivation area are low and, most of all, variable in the years seed yields [11]. Besides the breeding works leading to obtain new, more productive varieties researches are conducted also on effectivity of treatments which increase pea yielding e.g. provide plants proper supply with nitrogen by increase in symbiotic nitrogen fixation efficiency [8, 13, 24].

Interference at process of informative signals exchange between a pea plant and a bacteria is the important direction of action leading to earlier beginning and increase in symbiosis efficiency. [5, 14]. Conducted till now researches on symbiosis of papilionaceous plants and rhizobia discovered genetic background of this phenomenon and allowed identification of numerous plant and bacteria metabolites involved in the process of establishment of symbiosis and root nodules development [14, 16]. One of these compounds are flavonoids recognized as signal particles taking part in exchange of information between a plant and a bacteria [3], affecting level of nod genes activation [4, 7] and soils enzymes activity [21]. Moreover, flavonoids enlarge competition of rhizobia what concerns not only rhizobia introducing to the soil in a form of inoculum but also autochthons present in the soil [7]. Signal compounds (flavonoids, nod factors and others frequently undergo the processes which cause the decrease in their concentration on the way from producers to the target organism [12]. It can be assumed then that their insufficient amount is a cause of low effective symbiosis, what considerably limits plant supply in nitrogen and decreases their yielding. Therefore, application of flavonoid compounds to the soil together with seeds can increase the amount of nodules, and by this way can improve the growth of plants.

The aim of researches was to evaluate the effect of flavonoid extract, obtained from germinating seeds on pea ontogenesis and forming of physiological indices of growth and yielding.

2. Material and methods

The researches were conducted at greenhouse of Institute of Soil Science and Plant Cultivation – State Research Institute in Puławy, in Mitscherlich pots containing a mixture of soil and sand in amount of 5 and 2 kg, respectively. Before the sowing pea seeds were soaked in the distilled water (H_2O) or in flavonoid extract (F) with a concentration 10µM. It was used 100 ml of preparation F or water on 1 kg of seeds. Flavonoid extract was obtained from germinating seeds of pea according to method described in details in paper of Kidaj et al. [6]. Pea seeds were sterilized by immersion in 0.1% $HgCl_2$ for 3 min, rinsed with sterile distilled water, then treated with 70% ethanol for 3 min, followed by a sterile water wash. Then, the seeds were shaken in sterile water, in darkness, for 4 days at 28°C. After removal of the sprouted seeds, the supernatant was extracted with ethyl acetate in the ratio 10:1 (v/v). Ethyl acetate was evaporated and the pellet was resolubilized in 95% ethanol and stored at 4°C [4]. The amount of the flavonoids was determined by drying and weighing the ethanol extract. The approximate flavonoid concentration in seed exudates was calculated in relation to the molecular weight of authentic flavone [7].

5 plants of pea var. Medal grew in each pot An experiment was established at completely randomized design, at 3 replications. In whole vegetation period soil moisture was kept on the level of 60% field water capacity. To watering of plants was using a precise device equipped with a computer which controls the amount of water served to the pots. The dynamics of emergences was evaluated every day by counting the appearing seedlings in the pots up to time after which there were any new plants at them. Only normal germinating seeds and fully developed seedlings were taking into consideration during the counting of emergence index.

In the aim of evaluation of mass increase dynamics pea plants were harvested in the 3 developmental phases: BBCH 65 - full flowering, 50% of flowers open, BBCH 75 - 50% of pods have reached typical length and BBCH 98 - fully ripe, all pods dry; seeds dry and hard. During the each harvest the fresh and dry mass of particular organs were determined. Mass of roots was determined when they were washed on the dense metal sieves. Dynamics of mass increase was evaluated on the base of relative growth rate (RGR) with using a formula of Evans [1]:

$$RGR = (\ln W_2 - \ln W_1)\,(T_2 - T_1)\ [g\ (g\ day^{-1})^{-1}],$$

where:
W_1 – dry matter at the beginning of measurement period
W_2 – dry matter at the end of measurement period
T_1 – beginning of measurement period
T_2 – end of measurement period.

Moreover, in the period of flowering (BBCH 60) and green pod (BBCH 75) the nitrogenase activity was measured and number and dry mass of nodules were established. In this aim pea roots were dug from the topsoil (25 cm), washed and put into the calibrated and airtight closed bottles. Then from each bottle 10% of gas phase capacity was removed and in this place the same capacity of acetylene was injected. After incubation period (2 hours) 1 ml of mixed gas phase from bottles was taken with the use of tight syringe and injected to the gas chromatograph CSI (Cambridge Scientific Instrument Co, UK), which was equipped with FID detector (Flame-Ionization Deterctor). Deoxygenated and dewatered nitrogen with concentration of oxygen and H_2O below 2 ppm and purity 5.0 was used as a buoyant gas. As a measure of nitrogenase activity was recognized the amount of ethylene reduced from acetylene in effect of this enzyme action. A standard of ethylene with concentration 100 ppm in air was used in analyze (Fluka, Germany).

During the harvest at full maturity seed yield and some elements of its structure were additionally determined (number of pods, number and mass of seeds and their humidity). Results of research, as a means from 3 pots, were elaborated statistically. The semi-interval confidence of Tukey at level of significance a = 0,05 was used in analysis of variance. Program Statistica v. 10.1. was used to statistical calculations

3. Results

The emergences of pea plants occurred after 8 days from sowing. With amount of high seeds quality (germination capacity 94%) a very good dynamics and uniformity of germination were obtained. It was found that preparation F had not effect on course of pea plants emergence. It can be assumed that flavonoid compounds which are the carriers of molecular signal emitted by a host-plant in way of bacteria do not stimulate a seed germination as Nod factors which are the carriers of such signal from the bacteria side [6, 17, 18, 26].

Application of flavonoid preparation had an effect on mass accumulation by pea plants (tab. 1), however the biggest differences of dynamics of above-ground weight increase was found in the period from sowing to the flowering (26,3%), while in the case of generative organs mass from flowering to green pods phase (11,6%). It is worth to underline, that weight of stems, leaves and seeds of pea was increasing up to full maturity phase, while roots mass was considerably lower at the end of ontogenesis than at phase of flowering and green pod. The same tendency was observed in the case of nodules, whose number in green pod were lower than at flowering. Loss of roots weight is known in agriculture and described by some authors [15, 27]. While the connection between preparation F use and increase of vegetative and generative organs mass, even at the end period of their growth and development, can point out a better nitrogen nutrition of plants which grew from seeds dressed with a flavonoid preparation. Nitrogen concentration in plants was increasing as a result of nodules number and weight increase [20].

Table 1. Relative growth rate (RGR) of pea plants [g (g · day^{-1})$^{-1}$]

Soaking of seeds	Developmental stages of plants (BBCH)		
	00–60	60–75	75–89
Above-ground part			
H_2O	0.57	1.64	1.35
F	0.72	1.83	1.44
$LSD_{0.05}$	0.14	0.17	n.s.
Roots			
H2O	0.36	0.85	-1.14
F	0.58	0.99	-1.18
$LSD_{0.05}$	0.16	n.s.	n.s.

n.s. – differences not significant

Source: own work

Soaking of seeds in the flavonoid preparation had a positive effect on analyzed parameters of pea nodules during flowering (BBCH 60) (tab. 2). The increase of nodules number per plant in effect of this treatment using was greater than the increase of nodules weight per plant. The increase of nodules number in effect of seeds soaking with the flavonoid extract amounted to 19.1%, and the increase of nodule weight- to 13.6%, while mass of single nodule did not change significantly.

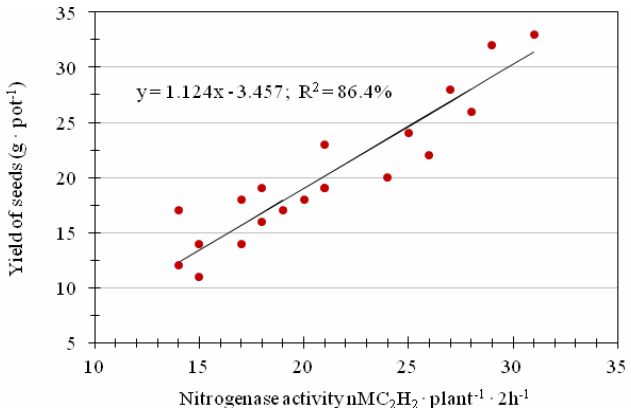
In the phase of green pod (BBCH 75) it was found the lowering of nodules number and increase in their weight what caused the increase of single pea nodule mass. An effect of using of preparation F on number and mass of nodules per pea plant was greater in the period of flowering than in the pod setting. It can resulted from the fact that, intensity of nitrogen fixation is greater in the flowering period compared to the remaining phonological phases of pea plants. Flavonoids excreted by various plant species cause induction of genes nod in different bacteria groups. For instance clover or pea excrete luteolin and naryngenein, and soybean daidzein and genistein [2]. Moreover, also concentration of these solutions has a great significance [10]. The excretion from germinating seeds contains frequently the mixture of these compounds, so its efficiency in relation to induction of nod genes which code a process of nodule formation is in general greater than efficiency of single flavonoid compounds [4].

The decrease in nodule number and its mass per 1 plant from period of flowering to the green pod period was an effect determinating a way of pea nodules development, which are counted to so called "indeterminated nodules". In this kind of nodules the meristem is active through the whole time of its development, thank to its new cells which still grows in nodules, and it results in continuous enlargement of size (particularly length) and weight of these organs [22, 23].

It was found also a significant effect of a preparation F on intensity of pea nitrogenase. This effect was greater in the flowering period than pod setting because in the flowering the activity of nitrogenase was greater of about 36% than in the period of green pod.

As a result of changes found at growth and development of pea plants caused by use of F preparation it was the increase of seed yield and improvement of its structure features (tab. 3). The increase of seed yield in effect of this treatment amounted to 10.7%, but the value of this effect was greater in the green pod period (BBCH75) than in the full maturity (BBCH 98). In the conducted experiment it was found a great dependency between the number and weight of nodules and pea yield, what indicates that number of "places of molecular nitrogen reduction and symbiotic exchange" which are single root nodules directly proportional translates into the size of yield, and the enlargement of these places on plant roots in effect of flavonoid preparation activity can improve their yielding [7]. The results of Voisin et al. [24] research, in which was showed a close relationship between root nodules biomass and obtained seed yield are the confirmation of this reasoning.

The conducted researches showed also a close relationship between nitrogenase intensity and seed yield (Fig. 1). It can be then assumed that enlargement of number and mass of root nodules affects positively also an activity of this enzyme. A confirmation of this conclusion can be found in research results of Niewiadomska et al. [9], which showed such dependency in relation to alfalfa.



Source: own work

Fig. 1. Relationship between number of root nodules and seed yield

Table 2. Number and dry matter of root nodules and nitrogenase activity of pea plants

Description	BBCH 60		$LSD_{0.05}$	BBCH 75		$LSD_{0.05}$
	H_2O	F		H_2O	F	
Number of root nodules per plant	64.5	76.8	6.4	60.7	62.4	n.s.
Dry matter of root nodules (mg per plant)	70.4	85.0	4.2	78.1	88.8	2.6
Dry matter of 1 nodule (mg)	1.09	1.11	n.s.	1.29	1.42	0.10
Nitrogenase activity ($nMC_2H_2 \cdot plant^{-1} \cdot 2h^{-1}$)	20.4	28.6	3.7	16.5	19.6	2.1

n.s. – differences not significant

Source: own work

Table 3. Yield of pea seeds and their components

Description	BBCH 75		$LSD_{0.05}$	BBCH 89		$LSD_{0.05}$
	H_2O	F		H_2O	F	
Number of pods per plant	7.1	9.2	1.1	5.6	6.0	0.2
Number of seeds per pod	3.3	3.2	n.s.	4.07a	3.70a	n.s.
Number of seeds per plant						
Weight of 1000 seeds (g)	92.3	94.1	n.s.	224	218	n.s.
Yield of seeds (g per pot)	7.4	9.1	1.4	20.8	22.1	2.6

n.s. – differences not significant

Source: own work

4. Conclusions

1. Soaking of seed in the flavonoid preparation positively affected mass accumulation by pea plants. The greatest differences at dynamic of vegetative organs weight increase were found in the period from sowing to flowering, and generative organs – from green pod to full maturity.

2. Use of flavonoid extract had profitable effect on number and mass of root nodules and nitrogenase intensity, what in the consequence caused a significant increase in pea yield. The effect of this preparation on microbial parameters was greater in the period of flowering than pod setting.

3. The increase in pea seed yield in effect of this preparation use was a consequence of enlargement of pods density per plant, number of seeds per plant and better filling of seeds.

4. Use of a flavonoid preparation can be an important element of ecological method of improving symbiotic nitrogen fixation efficacy, and by this – increase in pea yield.

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