EFFECT OF NARINGEIN AND QUERCETIN ON ACTIVITY OF *NODABC* GENES OF STRAIN D293 AND FOLLOWING NODULATION AND NITROGEN FIXATION RESPONSE OF INOCULATED PEA PLANTS (*PISUM SATIVUM* L.)

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Summary. Concentration and time course dependence of two structurally related plant flavonoids naringenin and quercetin on the *nod*ABC gene transcriptional activity (as reporter gene *nodC-lacZ* activity) in *Rh. leguminiosarum* bv. *vicae* strain D923 and respective growth, nodulation and nitrogen fixing responses of plant host (garden pea) to inoculation with pre-induced strains were studied. Naringenin was more effective as a common *nod* genes inducer with maximum activity obtained at 0.5μM, while quercetin showed lower activity at a concentration of 15μM. According to the results quercetin was the better common *nod* genes-suppressor of *Rh. leguminosarum* bv *viciae* D293 growth. Incubation of germinating pea seeds with flavonoids–preactivated inocula (applied in effective concentrations), resulted in changed nodulation and nitrogen fixing patterns of plants after 35 days of growth. No direct relationship between common nod gene transcriptional activity of activated rhizobial inocula, following nodulation and nitrogen fixing response of plants was observed.

Key words: flavonoids, *nod* genes, nodulation.

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INTRODUCTION

Flavonoids of leguminous plants act as chemoattractants, growth regulators towards symbiotic soil microorganisms, and as signals involved in symbiotic interactions (Shirley, 1996). The main role of flavonoids in the initiation of symbiosis is to activate the protein product of the regulatory *nodD* gene of bacterial symbiont (Chen et al., 2005). This *nodD* protein controls the regulation of *nodABC* regulon necessary for the synthesis of carbon backbone of bacterial *Nod* factor triggering nodule morphogenesis (Schultze and Kondorosi, 1998). Regulation of *nodD* gene differs among the rhizobial species. In *Rh. leguminiosarum* bv. *viciae,* where only one copy of the gene exists, the effect of flavonoids on *nodD* gene is strongly affected by the molecular structure of flavonoid co-inducers (Long, 1989). On the other hand, flavonoids have been shown to affect the nodulation and nitrogen fixation and the pre-treatment of the inocula with certain flavonoids helped to overcome some limiting for nodulation and nitrogen fixation environmental conditions (Zhang and Smith, 1995; Begum et al., 2001). In a previous work it was found that maximum positive effect of naringenin and quercetin on the activity of common *nodABC* genes was obtained at concentrations between 0.5 and 15 μM, during 24 h cultivation (Tsvetkova and Georgiev, 2004). Scarce information about the influence of positively or negatively activated *nodABC* genes of the strain on subsequent nodulation and nitrogen fixation of the inoculated pea plants is available which motivated the aim of the present work.

MATERIALS AND METHODS

Rhizobium leguminosarum bv *viciae*, strain D923, carrying pIJ1477, a plasmid with *nodABC- lacZ* fusion, allows determination of *nod* gene activity as *β*-galactosidase activity (E.C. 3.2.1.23) (Miller, 1972). The strain was cultivated on a liquid YEM medium at 28°C on a rotary shaker (120 rpm). Naringenin (from Sigma-Aldich) at concentrations of 0.5 μM was used as *nod* gene inducer. Quercetin (from Fluka) at concentrations of 15 μM was used as *nod* gene suppressor (Firmin et al., 1986). Samples for analysis were collected 24 h later. Protein content of *Rh. leguminosarum* cells was determined according to Bradford (1976). Bacterial growth was estimated after measurement of absorption of the bacterial suspension at 600nm and presented as colony forming units (CFU). The three-day-old seedlings of pea (*Pisum sativum* L. cv. Bohatyr) were inoculated with *Rh leguminosarum* bv *viciae* strain D293. Prior to plant inoculation, bacteria were incubated with 0.5 μM naringenin and 15 μM quercetin for 24 h. The infected seedlings were transferred to 1.2 L pots (two plants per pot) containing nutrient solution without mineral nitrogen (Yamagishi and Yamamoto, 1994) and were grown in a green house

under natural light and heat. Plants were harvested on the 35th day after germination. Nodules were submitted to acetylene reduction assay (Hardy et al., 1973). Statistical analysis of the means was performed using the Fisher LSD test ($Pd \leq 0.05$) after performing a multifactor ANOVA analysis.

RESULTS AND DISCUSSION

The incubation of strain D293 with naringenin decreased the overall cell growth, but increased *nodABC* transcriptional activity under laboratory cultivation conditions (Fig. 1) (Tsvetkova and Georgiev, 2004). Quercetin applied in the same concentration range as the *nod* gene effector resulted in only 3-fold increase in β-galactosi-

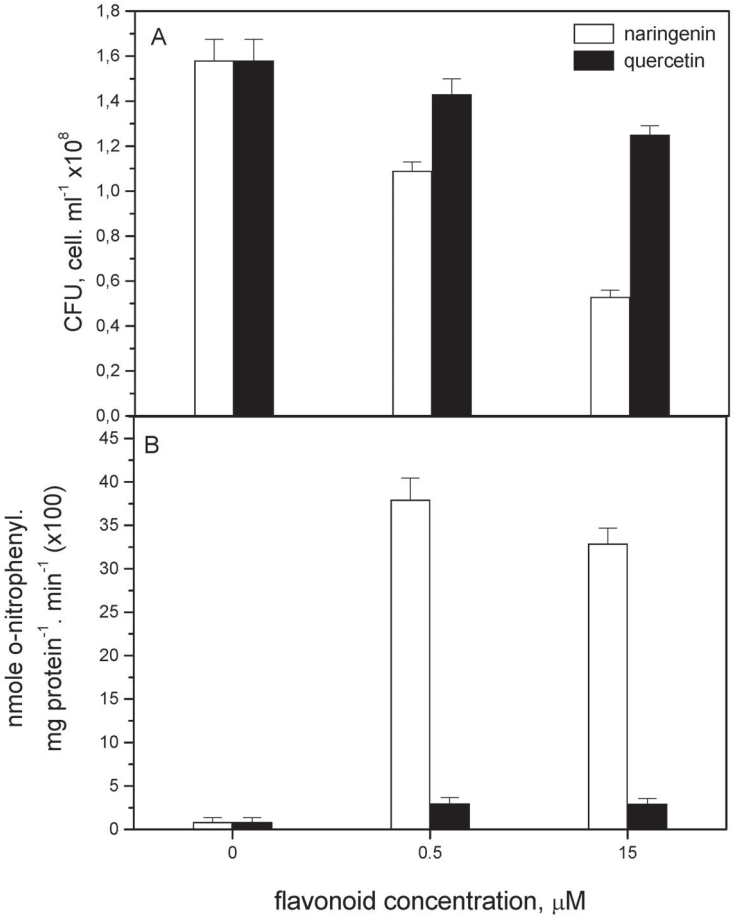

Figure 1. Effect of naringenin and quercetin on bacterial growth (A) and *nod* gene expression (B) of *Rh. leguminosarum* bv *viciae*, strain D293. Control: flavonoid non-treated bacteria.

dase activity, which could be due to a decrease in cell division, represented as CFU, after 24 h growth. It became obvious that the effective concentrations of the studied flavonoids for cell growth and for their nod gene transcriptional activity varied. The compounds studied are structurally related and differ in the number of -OH group substituted in the A, B and C rings of their molecule (Long, 1989, Tsvetkova and Georgiev, 2004). This could be the reason for naringenin higher activation of the genes (12.5-fold) compared to quercetin (Begum et al. 2001, Tsvetkova and Georgiev, 2004).

Inoculation of pea seedlings with material carrying diversely induced *nodABC* genes with different cell concentrations resulted in altered patterns of nodulation and nitrogen fixing activity of the host after 5 weeks of growth (Table 1). Dry matter accumulation in plants increased (129.8 % of control) when inoculum was pre-treated with 15μM quercetin. Dry matter accumulation did not change when 0.5μM naringenin pre-treated inoculum was used. "Naringenin" plants formed less but heavier nodules with decreased specific nitrogenase activity compared to the control. "Quercetin" plants indicated higher nodule number and dry weight but did not exhibit any changes in specific acetylene reduction rate as well (Table 1). Thus, the positive effect on the host dry weight after inoculation with quercetin pre-treated bacterial strains, was most probably due to other developmental growth changes. The contradiction between the positive effect on nodulation and DW of host plants and the negative influence on nitrogenase activity observed at day 35, could suggest accelerated aging of symbiotic apparatus provoked by the treatments (Hirsh and Smith, 1987). Positive relationship between nodulation patterns and accumulated DW per plant can be regarded as an evidence for effective nitrogen nutrition. Accumulation of certain degradation products from flavonoid metabolism inside the cells could cause the nodule aging observed after the treatments (Rao and Cooper, 1994). Further experiments are necessary to elucidate this problem.

Table1. Effect of inoculation with naringenin and quercetin pre-treated cells of *Rh. leguminosarum* bv *viciae* on plant biomass accumulation, nodulation and acetylene reduction rate of 35-day-old pea plants.

Variants of inoculum's pre-treatment	Total plant dry weight (g. plant^{-1})	Nodule number per plant	Nodule dry weight (g. plant^{-1})	SAR μM C_2H_2. gFW^{-1}. h^{-1}
Control - non-treated inoculum	0.47 ± 0.02^a	216 ± 35^{ab}	0.017 ± 0.001^a	10.42 ± 0.83^b
Inoculum + 0.5μM Nar	0.46 ± 0.03^a	185 ± 27^a	0.018 ± 0.001^a	8.3 ± 0.41^a
Inoculum + 15μM Quer	0.61 ± 0.01^b	245 ± 17^b	0.02 ± 0.001^b	10.5 ± 0.72^b
LSD (0.05)	*0.04*	*54.63*	*0.002*	*1.35*

Acknowledgements: This study was supported by "Progress in plant investigations for the improvement of sustainability of agriculture (PISA-INI14/01.09.2005)" Project (Bulgarian Ministry of Education and Sciences).

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