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Pleiotropic Effect of Fluoranthene on Anthocyanin Synthesis and Nodulation of *Medicago sativa* is Reversed by the Plant Flavone Luteolin

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The symbiosis between leguminous plants e. g. vetch, beans, alfalfa, and soil bacteria of the genus *Rhizobium* is of considerable agronomical importance, since it results in the fixation of atmospheric N_2 in the order of 2-400 kg ha^{-1}. Factors that affect this symbiosis are therefore of great economic importance. Numerous environmental signals, e.g. UV-light, heat drought stress, wounding, nutrient deficiency and the application of heavy metals have been shown to influence the formation of nodules on leguminous roots (Vincent 1965; Porter and Sheridan 1981).

Recently it has been found, that polycyclic aromatic hydrocarbons (PAHs; e.g. anthracene, phenanthrene, fluoranthene), which occur as ubiquituos environmental contaminants due to the combustion of fossile fuels, can inhibit nodulation of *Medicago sativa* (Wetzel *et al.* 1991). Fluoranthene is one of the dominant PAHs found in urban particulate matter, sewage sludge or beside motorways (Jones 1988). Several organisms have been shown to be able to metabolize and mineralize fluoranthene (Weißenfels *et al.* 1992; Foght and Westlake 1988) but the uptake of fluoranthene is limited due to low solubility of fluoranthene in water and strong adsorption to humic substances in soil (Weißenfels *et al.* 1992). *Rhizobium meliloti* cannot degrade fluoranthene (Brandt 1989). Toxic effects of fluoranthene on bacterial growth have never been observed. In contrast to their rhizobial symbiotic partners, alfalfa plants grown on a solidified fluoranthene-containing medium, exhibited symptoms of toxicity. They showed a dose-responsive decrease in shoot length and, if inoculated with *R. meliloti*, inhibition of nodule formation (Wetzel *et al.* 1991). Growth retardation is accompanied by a decrease in anthocyanin pigmentation of shoots, and an atypical accumulation of anthocyanins in roots. Synthesis of anthocyanins proceeds via the phenylpropane pathway as does synthesis of flavonoids. The major difference between the biosynthetic pathway for anthocyanins and that for flavones (flavonoids) is the reduction of the 4-carbonyl in the former, followed by water abstraction (Hradzina, 1982). Plant flavonoids are known to play a central role in the signal exchange of the Legume-*Rhizobium* symbiosis. Seeds of leguminous plants, as well as the developing roots of seedlings, exude several flavonoids that trigger complementary mechanisms for the establishment and development of root nodules. The best documented function of flavonoids is the induction of certain rhizobial nodulation (*nod*) genes (Fisher and Long 1992). The products of the *nod* genes are involved in the synthesis of Nod factors, which in turn initiate hair curling and cell division in the inner root cortex of the host plant (Truchet *et al.* 1991). As outlined above,

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phenylpropane derived compounds and flavonoids have been implicated in nodule development. Since fluoranthene impairs nodulation and induces the production of anthocyanins, it is possible that these events are causally linked via phenylpropanoid metabolism. In order to test this hypotheis, we tried to overcome the inhibitory effects of fluoranthene by exogeneous application of the flavonoid luteolin. Luteolin is usually exuded from alfalfa seeds and responsible for *nod* gene induction in *R. meliloti* (Hartwig *et al.* 1989). In this paper it will be demonstrated, that luteolin antagonizes the fluoranthene mediated inhibition of nodule formation and prevents the accumulation of anthocyanins in roots. These findings with respect to rhizobial *nod* gene regulation and plant developmental physiology will be discussed.

MATERIAL AND METHODS

Plant culture: seeds of *Medicago sativa* (alfalfa) cv Du puits (purchased from Ruppersberg, Cölbe, Germany) were washed with 0.1% Tween 20 for 3 min, rinsed several times and then surface sterilzed with 30% hydrogen peroxide for 10 min. These procedures were carried out in a sonication bath. After sterilization, seeds were washed ten times with sterile tap water and then dried under a laminar flow hood. Seeds treated in this way could be stored sterile for several weeks without any decrease in germination rate.
Sterilized seeds were germinated on NB (nutrient broth, Difco laboratories) agar for two days in a growth chamber and then transferred to a mineral medium (modified from Broughton and Dilworth 1971) solidified with Gelrite®. The pH was 6.8 and the medium was supplemented with 0.5 mM KNO_3. Light and humidity conditions were as follows: 16 h, 25° C light period / 8 h, 20° C dark period.
Fluoranthene and luteolin treatments: fluoranthene (10 µM; purchased from Serva) and luteolin (4 µM; purchased from Roth) were added to the mineral medium from stock solutions in N'-N-dimethylformamide (DMF) prior to pouring the medium into petri dishes. Alternatively fluoranthene (0.5 to 100 µg) and luteolin (15 µg) were sprayed as solutions in diethyl ether onto solidified medium (Wetzel *et al.* 1991). Luteolin and fluoranthene were tested either in combinations or alone. Control plates were with either DMF or diethyl ether, as appropriate.
Plant inoculation: Rhizobium meliloti MV II grown overnight in 20 E liquid medium (Werner *et al.* 1975) to mid log phase, was diluted to a concentration of 10^8 cells x ml^{-1} and used to inoculate solid mineral medium (50 µl per dish). Where fluoranthene and luteolin were added as etheral solutions, inoculation was carried out 24 h before spraying.
Five petri dishes with 3 plants each were scored for each treatment. The time course of nodulation was followed by counting total nodule number per petri dish at 15, 20 and 25 d after inoculation. Statistical analyses were done using Student's T-test.
Quantification of anthocyanins in root and shoot extracts: plants were harvested 20 d after inoculation, separated into shoots and roots, and then weighed. Afterwards plant material was dried at 60° C overnight, weighed again and fragmented, using a micro-dismembrator (Braun-Melsungen, Germany). Extraction of plant tissue was carried out with acidic methanol (65% methanol adjusted to pH 1 with HCL) for 15 min in a water bath at 55° C (Given *et al.* 1988). After centrifugation (11 600 g), the supernatants were collected and tissue extraction was repeated as above. Supernatants were pooled and then divided into two aliquots. One aliquot was adjusted to pH 5 with 100 µl of 10 % NaOH. All samples were

made up to a volume of 1 ml with 65 % methanol. The difference in absorption at 520 nm between the red coloured sample (pH 1) and the green coloured sample (pH 5) was measured. Anthocyanin concentration was calculated, using the molar extinction coefficient of 36,000 (Wrolstaat *et al.* 1970).

RESULTS AND DISCUSSION

Anthocyanin content of shoots and roots following fluoranthene treatment: fluoranthene caused a decrease in anthocyanin content of shoots when applied as an etheral solution to the surface of solidified medium (0.5 µg per petri dish) or at a concentration of 10 µM dissolved in the mineral Gelrite medium. Higher concentrations (5 µg per petri dish) caused a further decrease in anthocyanin of shoots, but roots showed intensive red pigmentation due to the accumulation of anthocyanins (figure 1). Based on its characteristic absorption spectrum (data not shown), the red pigment in roots could definitely be identified as anthocyanin, but not yet further specified. The anthocyanin accumulation became apparent 7-8 days after transfer of seedlings to fluoranthene containing medium and remained present until plant harvest (15 days after transfer). Loss of anthocyanin pigmentation of shoots could be reversed by transferring plants to a medium without fluoranthene. However, anthocyanin pigmentation of roots was not affected by this transfer. Changes in anthocyanin content following fluoranthene application were observed in alfalfa with and without R. *meliloti* inoculation.

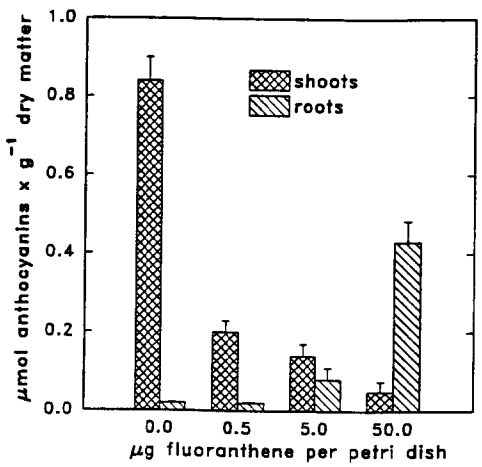

Figure 1. Anthocyanin content in roots and shoots versus fluoranthene concentration. Bars represent the mean of 6 experiments at each concentration. Each experiment consists of three plants per petri dish 15 d.p.i. Error bars indicate standard deviation.

Table 1. Effects of fluoranthene on nodulation and shoot growth of *M. sativa*, 15 days after inoculation with *R. meliloti*. (Values followed by different symbols are significantly different at the 0.05 level)

	Fluoranthene concentration (µg per petri dish)							
	0.0	SD*	0.5	SD	5.0	SD	50.0	SD
Nodules per plant	7.3[a]	2.9	4.4[b]	2.4	2.0[c]	1.9	1.5[c]	1.4
shoot (mg) dm**	62.0[a]	2.6	60.3[a]	3.4	53.8[b]	3.4	28.9[c]	2.6
shoot length (mm)	42.0[a]	5.3	42.8[a]	7.5	39.6[a]	7.5	22.7[b]	2.5

*Standard deviation of the mean **dry matter

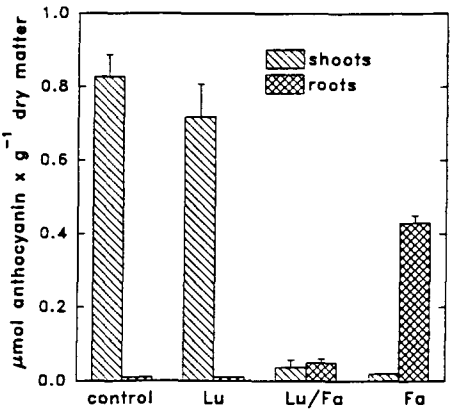

Figure 2. Anthocyanin content 20 d.p.i. in shoots and roots of plants treated with luteolin, fluoranthene and luteolin/fluoranthene coapplication. Bars represent the mean of 6 experiments of three plants at each application procedure. Error bars indicate standard deviation. Spray application: 25 µg fluoranthene and 15 µg luteolin per petri dish; mix application 10 µm fluoranthene, 4 µM luteolin

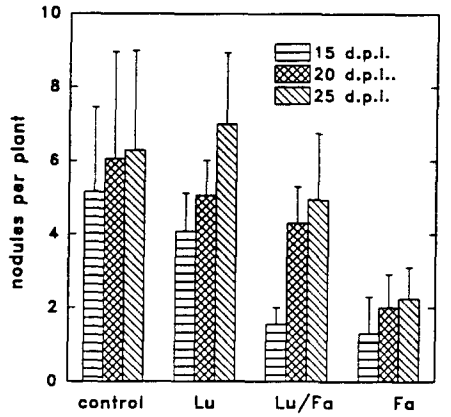

Figure 3. Nodulation kinetic (15,20 and 25 d.p.i. of plants treated with luteolin, fluoranthene and luteolin/fluoranthene coapplication. Nodulation is expressed as number of nodules per plant. Bars represent the mean of 6 experiments at each application procedure. Each experiment was carried out with three plants per petri dish. Error bars indicate standard deviation.

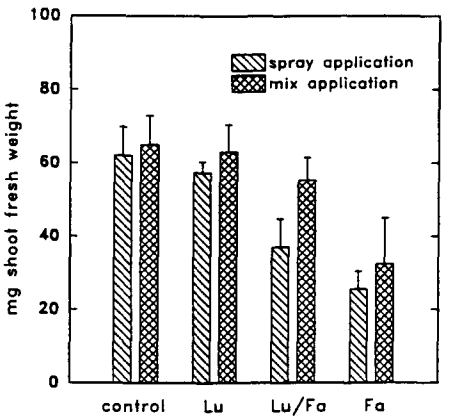

Figure 4. Shoot weight per plant (20 d.p.i.) in response to application of luteolin, fluoranthene and luteolin/fluoranthene coapplication. Concentrations of luteolin and fluoranthene see figure 2. Bars represent the mean of 6 experiments at each application procedure. Each experiment was carried out with three plants per petri dish. Error bars indicate standard deviation.

Effect of fluoranthene on nodulation and plant growth: fluoranthene caused a dose-responsive delay and decrease in nodulation of alfalfa seedlings beginning from 0.5 µg per petri dish. A total inhibiton of nodulation occured at a concentration of 100 µg per petri dish. Toxic effects on the growth of bacteria can be excluded. The visual appearance of the colonies was normal compared to the non-treated petri dishes.

The process of nodulation was more sensitive to fluoranthene than the reduction in shoot growth. The reduction in shoot length and shoot weight became significant when the fluoranthene concentration exceeded 5 µg per petri dish (table 1). Toxic effects of fluoranthene on shoot growth and nodulation could partly be reversed, when plants were transferred to medium without fluoranthene. Shoot growth recovered to control levels and shoots showed new anthocyanin pigmentation but plants failed to nodulate unless they were reinoculated following fluoranthene removal. Plants inoculated and grown for up to five days on medium

lacking fluoranthene also showed reduced nodulation when transferred to fluoranthene containing medium. There was a tendency towards an increase in lateral root formation in fluoranthene treated plants. However due to high standard deviations, differences between fluoranthene treated and non-treated plants were not significant on the $p = 0.05$ level.

Effects of exogeneously applied luteolin: luteolin was applied together with fluoranthene either as etheral solution (15 µg per dish) or at a concnetration of 4 µM to the mineral medium. Both treatments affected fluoranthene induced anthocyanin accumulation in roots. As shown in figure 2, luteolin prevented anthocyanin accumulation in roots but had no effect on loss of anthocyanin pigmentation from shoots. Since luteolin exhibits *nod* gene-inducing activity, we were interested, whether it could antagonize the fluoranthene induced depression of nodule formation. In figure 3 the suppression by luteolin of the fluoranthene mediated inhibition of nodulation is documented. This suppressive effect was time dependent and first became visible 15 days after inoculation. Twentyfive days after inoculation, plants receiving both fluoranthene and luteolin had just as many nodules as the untreated control plants. Shoot growth was almost comparable with that of control plants (figure 4).

Summarizing our observations, we found that fluoranthene causes a significant depression of nodule formation in the symbiosis of *M. sativa* with *R. meliloti*. This was accompanied by a dramatic accumulation of anthocyanins in the roots of *M. sativa*. Both effects could be reversed by exogeneous application of luteolin. Two possible explanations (A and B) for these observations will be discussed.

(A) **By influencing phenylpropane metabolism, fluoranthene leads to a lack of nod-gene inducing flavonoids, for example luteolin, in the rhizosphere**. Luteolin is the first documented inducer of *R.. meliloti nod* genes released by alfalfa plants (Hartwig *et al.* 1989). As a result of *nod* gene induction in *R. meliloti* the nodulation factors (Nod factors; in 1990 the *Rhizobium* nodulation factor Nod Rm-1 was identified be Lerouge as a sulphated glycolipid) are produced by the bacterial symbiont, which in turn elicit root hair deformation and cortical cell division in the plant. Chemicals affecting the release or modification of *nod*-gene inducing flavonoids from alfalfa roots could influence the exchange of signals between host and microsymbiont. As shown in the present study, fluoranthene somehow interferes with phenylpropane metabolism, causing an excessive production of anthocyanins in root tissues. We propose, that this massive production of anthocyanins leads to reduced synthesis of other phenylpropane derived compounds, e.g. the *nod* gene inducing flavonoids. The concentration of *nod* gene inducers in the rhizosphere of alfalfa has already been shown to limit nodulation. But a lack of *nod* gene inducing flavonoids in the alfalfa rhizosphere can be overcome by the exogeneous application of appropriate flavonoids (Kapulnik *et al.* 1987). We propose that exogeneously applied luteolin compensates for the lack of *nod* gene inducing flavonoids in the rhizosphere of fluoranthene treated plants. Moreover, luteolin not only recovered nodulation, but also increased shoot dry weight of fluoranthene treated plants to control levels, irrespective of whether they were inoculated or not. Due to this finding one has to consider additional physiological effects of luteolin directly beneficial for the plant.

(B) **Accumulation of anthocyanins in roots and the inhibition of nodulation in fluoranthene treated plants might be the consequence of a disturbed hormonal balance of the plant**. Until now toxic effects of fluoranthene on plant growth have not often been reported. Most authors used combinations of different PAHs in their studies, and did not distinguish between individual compounds. Gräf (1965) demonstrated growth promoting effects of PAHs on higher plants. In contrast Wagner and Wagner-Hering (1971) reported growth retardation. 3,4-Benzofluoranthene, at 6.2 $mg \cdot kg^{-1}$ soil caused a reduction of stem

length in wheat.

Here we report that fluoranthene (2mg · l^{-1}) reduced stem and fresh weight of *M. sativa*. Treated plants developed smaller leaves and shorter internodes than untreated control plants. There was also an earlier abscission of cotyledons (results not shown). Similar effects can be achieved by cutting off young roots and thus interrupting the acropetal transport of cytokinins from roots to shoot (Mohr and Schopfer, 1978). It is therefore possible, that fluoranthene influences the cytokinin transport and promotes accumulation of cytokinin in roots. The disturbance of the hormonal balance will not only diminish plant growth. Also inhibition of nodulation of alfalfa by *R. meliloti* following fluoranthene treatment might be explained by a fluoranthene mediated disturbance of the cytokinin/auxin ratio in alfalfa root cortical cells. Whether accumulation of anthocyanins in roots is also a consequence of a high cytokinin content in roots or is due to a disturbed phenylpropane metabolism, as argued before, remains to be elucidated. However, stimulation of anthocyanin production by cytokinins or cytokinin-like substances has often been reported (Pecket and Bassin 1974; Hradzina 1982). Conversely, treatments which increase anthocyanin levels, such as light or anaerobic growth conditions have been shown to influence cytokinin mediated processes, e.g. inhibition of adventitious root growth (Stafford, 1968) and diminishing of gravitropic curvature in light treated roots (Iversen and Siegel, 1976). It is generally accepted that phytohormone controlled processes are not dependent on the action of single hormones, but different hormones acting simultaneously. Growth and development of plants is triggered by cytokinins and auxins and the ratio of cytokinin to auxin concentration. Much attention in this respect has been paid to the effect of phenylpropane derived phenolic compounds on indoleacetic acid (IAA) oxidase, a peroxidase-type enzyme which is capable of auxin destruction. Anthocyanins, having a monohydroxy phenol B-ring, uniformly stimulate IAA-oxidase activity and thus have a potentially inhibiting effect on growth (Stenlid, 1976). Flavonoids with a catechol B-ring (luteolin) on the other hand inhibit IAA oxidase *in vitro*. This could explain the stimulatory effects of such compounds on plant growth. Moreover it has been suggested, that flavonoids might interfere with polar auxin transports (for references see Hirsch 1992). An auxin binding protein with a molecular weight of 23 kDa has recently been found in the symbiosome membrane of legume nodules (Jacobi *et al.* 1993).

Whether luteolin counteracts the toxic effects of fluoranthene directly or only reverses the effects of anthocyanins on the cellular auxin/cytokinin ratio needs further investigation. Beside the two explanatory models for the beneficial effect of luteolin discussed above there remains another explanation worth mentioning. We have to take into consideration, that toxicity of PAHs is also linked to the formation of oxygen radicals, as a result of cytochrome P-450 dependent hydroxylations (Babson *et al.* 1986; Southorn and Powis 1988). Flavonoids, on the other hand, have been shown to exhibit radical-scavenging activity (Torel *et al.* 1986). Although no hydroxylated fluoranthene metabolites have yet been found in alfalfa, (Wetzel, unpublished data) a radical-scavenging role for luteolin should not be disregarded.

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