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Regulation of isoflavone production in hydroponically grown *Pueraria montana* (kudzu) by cork pieces, XAD-4, and methyl jasmonate

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Abstract A mini-hydroponic growing system was employed for seedlings of kudzu vine (*Pueraria montana*) and contents of isoflavones (daidzein, genistein, daidzin, genistin, and puerarin) from shoot and root parts of seedlings were analyzed quantitatively. In addition, exogenous cork pieces, polymeric adsorbent, XAD-4, and universal elicitor, methyl jasmonate (MeJA), were used to regulate the production of these isoflavones. It was shown that cork pieces up-regulate the production of daidzein and genistein up to seven- and eight-fold greater than the levels obtained for control roots. In contrast, levels of glucosyl conjugates, daidzin and genistin, decrease up to five- and eight-fold, respectively. Cork treatment also induces the excretion of the root isoflavone constituents into the growth medium. Minimal levels of isoflavones are absorbed by the cork pieces. XAD-4 stimulates the production of glucosyl conjugates, daidzin and genistin, in root parts about 1.5-fold greater than that obtained in control roots. These are the highest amounts of daidzin and genistin that are observed (5.101 and 6.759 mg g^{-1} dry weight, respectively). In contrast to these two adsorbents, MeJA increases the accumulation of isoflavones in shoot rather than in root parts of seedlings, about three- to four-fold over control levels, with the exception of genistein. These studies reveal new observations on the regulation of isoflavone production in hydroponically grown *Pueraria montana* plants by two adsorbents (cork pieces and XAD-4) and MeJA elicitor.

Keywords Kudzu vine (*Pueraria montana)* · Secondary metabolite · Isoflavones · Cork pieces · XAD-4 · MeJA

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Introduction

Many edible legumes in the bean family (Fabaceae) are important sources of isoflavone secondary metabolites and soluble dietary protein (Kaufman et al. 1997; Kirakosyan et al. 2004). Our previous studies indicate that Japanese kudzu (*Pueraria montana*) turns out to have unusually high levels of genistein and daidzein isoflavones as compared with almost all other edible legumes examined (Kaufman et al. 1997; Lal et al. 2003). These two isoflavones and their respective glucosyl conjugates, genistin and daidzin, as well as another major isoflavone, puerarin, are produced mainly in the roots as compared with other organs of kudzu plants (Kirakosyan et al. 2003).

Knowledge of the processes that regulate the biosynthesis and localization of such bioactive isoflavones in plant cells is crucial to our understanding of the biological functions of these kinds of metabolites. For example, light treatment, as compared with dark treatment, results in significant increases in the levels of isoflavones in kudzu seedlings (Lal et al. 2003; Kirakosyan et al. 2003). There are also many useful biotic and abiotic factors that can influence the production of such secondary metabolites in root parts of plants. Fungal elicitors and modification of culture

conditions have been shown to increase root growth and secondary metabolite production in some cases (Lal et al. 2003). This may be attributed to both rhizosecretion from the roots to the culture medium as well as to the organ-specific localization of the desired secondary metabolites.

Many flavonoids are present exclusively in the cork layer and the dead bark part of the thickening roots (Hayashi et al. 1996; Yamamoto et al. 1996). Large amounts of flavonoid glycosides are also mainly distributed in the woody parts of thickening roots. For example, Yamamoto et al. (1992) report that flavonoid glycosides occur in the inner secondary xylem tissues of the root, whereas the free flavonoids are localized in the cork layers in roots of the leguminous plant, *Sophora flavescens*. The authors further show that in addition to lignin polymer, several lipophilic secondary metabolites of low molecular weight are accumulated in the dead cork tissues, whereas hydrophilic secondary metabolites tend to accumulate in the living cells of other parts of the roots (Yamamoto et al. 2001).

The main role of root-produced cork in nature is attributed to the regulation of water and mineral storage (Kolattukudy 1984) and to the chemical defense against pathogen attack (Kahl 1974). However, some other functions attributed to cork must also be considered. Recently, it was suggested that cork could be a universal stimulatory agent that increases the secondary metabolite production in several medicinal plants (Yamamoto et al. 2001). In addition, cork tissues increase the secondary metabolite production in various plant cell cultures in a manner that is different from the characteristic of the conventional elicitors (Yamamoto et al. 1992, 1996, 2001; Kirakosyan et al. 2001; Zhao et al. 2003).

Besides cork, some adsorbents are shown to stimulate biosynthesis of many secondary metabolites, in particular, anthraquinones and flavanones (Strobel et al. 1991; Yamamoto et al. 1996). Studies (Yamamoto et al. 1996, 2001) of flavanone biosynthesis in cell suspension cultures of *Sophora flovescens* show that, in addition to absorbing flavanones from the culture medium, both cork tissue and the chemical adsorbent, Diaion HP20, stimulate flavanone biosynthesis. In another study, Strobel et al. (1991) report that anthraquinones that are usually accumulated in the cells were successfully absorbed by polymeric adsorbents such as Amberlite XAD-2 or Wofatite ES concomitant with an increase in the overall anthraquinone productivity in *Galium vernum* cultures. Several investigators discuss different possible mechanisms of stimulation by these agents (Strobel et al. 1991; Yamamoto et al. 1996; Kirakosyan et al. 2001), but so far, they have not been verified. Earlier, we reported on the stimulatory effect of cork pieces on pseudohypericin biosynthesis in shoot cultures of St. John's wort (*Hypericum perforatum* L.) (Kirakosyan et al. 2001). The addition of the cork pieces (from 1 to 5 mg cork ml^{-1} of growth medium) slightly stimulates shoot growth and enhances pseudohypericin biosynthesis. Pseudohypericin production increases in proportion to the amount of cork material added within this concentration range. Further increases in the amount of cork pieces above 5 mg cork ml^{-1} inhibit both pseudohypericin production and shoot growth. We have also reported that organic and aqueous extracts of cork pieces did not affect the production of pseudohypericin (Kirakosyan et al. 2001).

In light of the above evidence, and our previous results on kudzu, we hypothesize that cork can stimulate the production of desired isoflavones in kudzu seedlings, which may then be released into the medium and absorbed by the cork tissue. We also compare the stimulatory effect of cork with polymeric adsorbent, XAD-4, and with the known universal elicitor, methyl jasmonate (MeJA).

Materials and methods

Plant materials and hydroponic cultivation of kudzu seedlings

Seeds of Japanese kudzu (*Pureraria montana*) were obtained from Adams-Briscoe Seed Company Inc., P.O. Box 19/325, East Second Street, Jackson, GA 30233-0019, USA. Kudzu seeds were germinated and grown in Bio Set^{TM} seed sprouting units obtained from Johnny's Selected Seeds Company, 184 Foss Hill Road, Albion, ME 04910-9731, USA. Seedlings in the Bio Set^{TM} units were watered once daily. When the first true leaves started to emerge from the seedlings, just above the cotyledons, the seedlings were transferred to a mini-hydroponic system composed of scintillation vials (20 ml capacity) that contain 12 ml full strength Hoagland's solution (Sigma Chemicals Co., St. Louis, MO, USA). Control and experimental environments were simulated by using a Precision Scientific Co. Dual Program Illuminated Incubator (model 818). To test the effects of cork, XAD-4, and MeJA on the production of isoflavones in kudzu, the incubators were programmed to provide continuous light (162 $\mu E\ m^{-2}\ s^{-1}$). The temperature of the incubator was maintained at 25°C.

The duration of experimental treatments was 15 days, at which time whole seedlings were harvested, washed with distilled water, separated into shoot and root parts, lyophilized, and ground into fine powders.

Well-dried recently harvested mature roots of kudzu were provided by Caroline J. Edwards, licensed soil scientist at Kudzu Connection, 722 Duncan Road, Rutherfordton, NC 28139, USA.

Preparation of cork pieces, XAD-4, and MeJA

New clean commercial corks (Fisher Scientific, Pittsburgh, PA) were used to cut 2-mm-thick cork pieces (50 g). These were fractionated into smaller pieces in a Waring blender. Fatty acids and pigments were extracted from these pieces with chloroform for 4 h at 70°C. The extraction was repeated until a colorless extract was obtained. The extract was separated from the cork matrix by filtering through Dacron filters and evaporated in a Büchi rotary evaporator. Polyphenols and low-molecular-weight compounds were extracted from dried cork material with methanol several times (each extraction for 4 h at 70°C) until a colorless

extract was obtained. After filtering, the methanol fraction was dried off in a rotary evaporator and the cork material was extracted with distilled water (three times for 8 h at 70°C) and autoclaved at 121°C (10 Pa) for 15 min. After filtering, the cork matrix was washed several times with deionized, distilled water and dried (as cited in Kirakosyan et al. 2001). The cork matrix obtained from this procedure was used in the experiments.

XAD-4 adsorbent (the same polystyrene non-polar polymeric adsorbent as XAD-2, however with lower average pore diameter, 40 Å vs. 90 Å) (Sigma) used in this study was washed with EtOH prior to the addition of Hoagland's solution. MeJA (Sigma) was directly added to and stirred in the Hoagland's solution.

In our studies, we used different concentrations of cork, XAD-4, and MeJA (3, 5, and 7 mg ml^{-1} of growth medium for cork and XAD-4, and 25, 50, and 100 μM concentrations for MeJA). An optimal concentration of cork pieces, and XAD-4 equal to 5 mg ml^{-1} of growth medium was chosen for further experiments. This is also based on the cork and XAD studies reported previously (Yamamoto et al. 1996; Kirakosyan et al. 2001). The 50 μM optimal concentration was used for MeJA.

Quantitative analysis of isoflavones and their glucosyl conjugates

All plant materials were dried and divided into shoots and roots. Triplicate samples, 0.2–0.5 g each, of the fine powder were prepared for each experiment. The powdered samples were extracted with 10 ml of 80% methanol at 50°C for 12 h, and an aliquot (10 μl) of the extract was analyzed by HPLC. In addition, lyophilized cork pieces, XAD-4 and powders of the growth medium were extracted in a same way as described above.

Experimental procedures to determine the isoflavone levels in cork layers and woody parts of mature kudzu roots were the same as described in Kirakosyan et al. (2003).

The HPLC analysis of isoflavones was carried out according to the methods described in Kirakosyan et al. (2003). The HPLC conditions were as follows: a Phenomenex Luna™ column (Torrance, CA 90501) (5 μm pore size, C-18, 150 mm × 4.60 mm), flow rate of 1 ml min^{-1}, Solvent A: water + 0.1% TFA (trifluoroacetic acid); Solvent B: acetonitrile + 0.1% TFA; HPLC running conditions consisted of a gradient of 5–100% B during a 30-min period; oven temperature was 40°C. A 10 μl aliquot of sample was injected onto a Shimadzu 10 AD HPLC system with a SPDM-10AV photodiode array detector (Shimadzu, Japan). Detection was set at 280 nm. The quantitative analysis of each compound in the extracts was analyzed by comparing it with the corresponding authentic samples of daidzein, daidzin, genistein, genistin, and puerarin obtained from Sigma-Aldrich Chemical Company, Milwaukee, WI 53201. Each peak was identified by the retention time and the characteristic UV spectrum.

Statistical analysis of data

Experiments were repeated at least three times and the data were analyzed statistically. All results are given as mean ± standard deviation (SD). Differences between variables were tested for significance by Student's *t*-test. A *P*-value of <0.05 was considered to be significant.

Results

Data from Table 1 suggest that the ratio of free isoflavones to glucosyl conjugates is higher in cork layers than in the woody parts. Thus, we hypothesize that exogenous cork pieces in this hydroponic growing system could play an important role as a regulator of biosynthesis, or as an absorbent of isoflavones. If this hypothesis proves to be true, then we would have a new way to grow roots, to stimulate the production of isoflavones, and to recover isoflavones from cork tissue or the growth medium.

For time-course analysis, we determined the isoflavone production in 3- and 7-day-old seedlings. However, isoflavone accumulation was not significant for 3- and 7-day-old control and elicitor-treated seedlings (data not shown), whereas significant differences were obtained after 15 days.

During co-cultivation of seedlings with cork pieces or with XAD-4 adsorbent, we observe different manners of interaction with kudzu seedling roots. Cork pieces easily float in the medium and directly interact with the roots, whereas XAD-4 adsorbent pieces remain at the bottom of the vials.

Our results show that cork stimulates the production of daidzein and genistein up to seven- and eight-fold, respectively, which is greater than the levels obtained for control roots (1.839, 2.683 and 0.235, 0.323 mg g^{-1} dry weight, respectively) (Fig. 1). However, we also find that the levels of glucosyl conjugates, daidzin and genistin, decrease up to five- and eight-fold, respectively (0.665, 0.551 and 3.810, 4.535 mg g^{-1} dry weight). Thus, the net accumulation of total isoflavones decreases by about 50% as compared to

Table 1 Comparison of contents of daidzein, daidzin, genistein, genistin, and puerarin (mg g^{-1} dry weight biomass) between the cork layer and the woody part of intact mature roots of kudzu (*Pueraria montana*)

Root parts examined	Daidzein	Daidzin	Genistein	Genistin	Puerarin
Cork layer of intact mature root	0.382 ± 0.064	1.282 ± 0.234	0.543 ± 0.075	2.248 ± 0.388	14.801 ± 0.923
Woody part of intact mature root	0.194 ± 0.033	4.951 ± 0.633	0.225 ± 0.035	3.377 ± 0.543	19.212 ± 1.023

$n = 3$; values are means ± SD

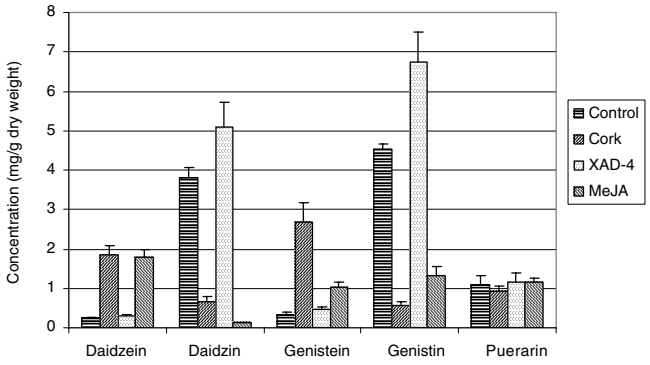

Fig. 1 Contents of isoflavones in root parts of control and elicitor-treated kudzu seedlings (mg g^{-1} dry weight biomass) ($n = 3$, error bars = ± SD; some error bars are not visible due to small standard deviation values)

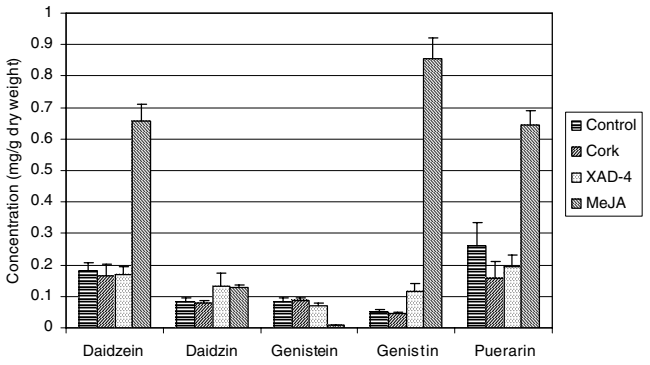

Fig. 2 Contents of isoflavones in shoot parts of control and elicitor-treated kudzu seedlings (mg g^{-1} dry weight biomass) ($n = 3$, error bars = ± SD; some error bars are not visible due to small standard deviation values)

that for control roots. In shoot parts of kudzu seedlings, no significant changes occur in levels of isoflavones. Taking these results into account, we show here that this kind of regulation with kudzu (*Pueraria montana*) differs from that observed for cork tissues by Yamamoto et al. (1996) with *Sophora flovescens* cell suspension cultures and Kirakosyan et al. (2001) with *Hypericum perforatum in vitro* shoot cultures, where cork pieces stimulate the net accumulation of prenelated flavonoids and polyketides, respectively.

Concerning XAD-4 and its stimulating effect, we find that this adsorbent stimulates the production of glucosyl conjugates, daidzin and genistin, in the root parts about 1.5-fold greater than that obtained in control roots (5.100, 6.759 and 3.810, 4.535 mg g^{-1} dry weight, respectively) (Fig. 1). However, no significant stimulation is observed for other isoflavone constituents. But, compared with cork pieces, its net stimulation of daidzein and genistein production is five- and seven-fold greater, respectively. Furthermore, the net accumulation of all isoflavones is 60% greater than that obtained for control. In contrast, no significant changes occur in levels of isoflavones in shoot parts of kudzu seedlings.

MeJA increases accumulation of all studied isoflavones in shoots about three- to four-fold over control levels, with the exception of genistein (Fig. 2). Net accumulation of all studied isoflavones is greater by about five times than that for control shoots. In roots, MeJA increases the production of daidzein and genistein over control levels (Fig. 1); however, total net isoflavone levels decreases by 50%.

In each of these studies, no significant changes were also observed for growth rate (1.7–1.9-fold net fresh biomass growth over 15 days treatment) in control, or cork-, XAD-4-, and MeJA-treated kudzu seedlings.

To determine the possible mechanisms of activation by these three stimulatory agents, we also examined isoflavone levels in intact cork pieces, XAD-4, and Hoagland's medium after the respective 15-day treatments. As seen from Table 2, the roots secrete a minimal amount of isoflavones into the medium. In the medium of control seedlings, we found only trace amounts of daidzein and genistein. Almost same amounts of these two isoflavones and puerarin were detected in the medium following XAD-4 treatment. The same is true for MeJA-treated medium. In contrast, exogenous cork pieces stimulate the exudation of all studied isoflavones from the roots to the medium. Here, the concentration of daidzein and genistein in the medium with cork is higher (0.101 and 0.158 mg g^{-1} dry weight, respectively) than for control treatment (0.034 and 0.018 mg g^{-1} dry weight, respectively). These results clearly show that cork stimulates the production of isoflavones in a manner different from that of the other two stimulatory agents.

A low concentration of all isoflavones is detected in intact cork pieces, indicating that cork can absorb these compounds from the growth medium. However, we did not detect any of these isoflavones in XAD-4.

Table 2 The contents of daidzein, daidzin, genistein, genistin, and puerarin in control, cork-, XAD-4-, and MeJA-treated residues of Hoagland's medium as well as in the added cork pieces and XAD-4 (mg g^{-1} dry weight biomass)

Fractions examined	Daidzein	Daidzin	Genistein	Genistin	Puerarin
Residue from control Hoagland's medium	0.034 ± 0.008	nd	0.018 ± 0.009	nd	nd
Residue from Hoagland's medium + MeJA	0.013 ± 0.004	nd	0.012 ± 0.005	nd	nd
Residue from Hoagland's medium + XAD-4	0.034 ± 0.009	nd	0.019 ± 0.003	nd	0.019 ± 0.004
Residue from Hoagland's medium + cork pieces	0.101 ± 0.075	0.031 ± 0.009	0.158 ± 0.096	0.006 ± 0.002	0.024 ± 0.003
Intact cork pieces	0.002 ± 0.001	0.008 ± 0.005	0.004 ± 0.003	0.008 ± 0.005	0.002 ± 0.001
XAD-4	nd	nd	nd	nd	nd

$n = 3$; values are means ± SD. nd, not detected

Discussion

Yamamoto et al. (2001) have outlined possible mechanisms for the stimulatory effects of cork on secondary metabolite biosynthesis. These mechanisms are classified as follows: (1) stress response of the cells by physical contact with cork; (2) prevention of further catabolism of metabolites through their absorption into cork tissue; (3) blocking of feedback inhibition by the products of biosynthetic enzymes through the removal of end-products from the biosynthetic sites; and (4) activation of biosynthetic enzymes that are responsible for the biosynthesis of these isoflavones.

Which mechanism is more likely in our case first requires some clarification. It is possible that we have a conversion of glucosyl conjugates of isoflavones to their respective aglycones which is activated through a stress response of the cells to physical or chemical contact with the cork pieces. It is interesting, in this connection, that the concentration of the known major isoflavone, puerarin, does not change in response to cork treatment. This compound is synthesized in the root parts of kudzu to a much higher extent than in the shoots, and remains almost at the same level for both control and elicitor-treated seedlings. Actually, we observe that shoot parts of seedlings, excluding MeJA-treated shoots, do not respond to this kind of stimulating effect; and in all cases, the seedlings produce almost the same levels of all the isoflavones studied (Fig. 2).

Based on our results, it is possible to account for different activation mechanisms by these stimulatory agents. XAD-4 did not absorb end-products such as isoflavones; thus, it is possible that this adsorbent changes the composition of Hoagland's solution through the absorption or exudation of some micronutrients. Finally, this can lead to the stimulation of biosynthesis of daidzin and genistin. This mechanism seems be more likely because we did not see any physical contact of XAD-4 absorbent particles with the roots.

Cork tissue is formed in the surface layers of stems and roots of higher plants, and presumably, its function is to act as a mechanical protector against environmental and biotic stresses. However, in newly grown kudzu seedlings, young roots do not form cork layers. Therefore, it is possible that exogenous cork pieces could effect the production of isoflavones through the absorption and removal of end-products from biosynthetic sites. This is presumed to be due to the lack of cork tissues, where the products are expected to accumulate *in situ*. However, we do not exclude other mechanisms of stimulation.

If this and other regulatory mechanisms can be elucidated in the future, we will have a more productive and feasible system to stimulate secondary metabolite productivity and exudation to the medium.

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