Journal of
Experimental
Botany

Genistein accumulation in soybean (*Glycine max* [L.] Merr.) root systems under suboptimal root zone temperatures

Feng Zhang and Donald L. Smith[1]

Department of Plant Science, Macdonald Campus of McGill University, Ste. Anne de Bellevue, Quebec, Canada H9X 3V9

Abstract

Genistein, as a plant-to-bacteria signal, plays an important role in the establishment of the soybean (*Glycine max* [L.] Merr.)-*Bradyrhizobium japonicum* nitrogen-fixing symbiosis. It is essential to the development of effective root nodules and responsible for inducing the *nod* genes of *B. japonicum*. Because suboptimal root zone temperature (RZT) delays infection and early nodule development, and decreases plant nodule number, and genistein addition overcomes some of this, it is reasonable to hypothesize that suboptimal RZT disrupts the inter-organismal signal exchange by inhibiting genistein synthesis. Four experiments were conducted to test these hypotheses. The results of these studies indicated that: (1) when soybean plants were germinated and maintained at RZTs ranging from 13 to 17 °C, root genistein concentration and content per plant were lower than those of plants with roots maintained at RZTs above 17 °C; (2) when plants were germinated at an optimal RZT (25 °C) then transferred to RZTs below 17 °C, and acclimated for a few days, root genistein concentration and content per plant were higher than those of plants with roots maintained either at optimal RZT, or transferred to RZT above 17 °C, although by the end of the experiment, the genistein concentration of root systems at below 17 °C RZT appeared to be declining to values below those of plants with above 17 °C RZT; (3) the root genistein concentration increased before the onset of nitrogen fixation and decreased thereafter; and (4) part of the effect of RZTs on genistein content per plant root system was from reductions in genistein concentration at lower RZTs, and part was due to decreased plant root growth.

Key words: Genistein, *Glycine max*, suboptimal temperature.

Introduction

The establishment of an effective nitrogen-fixing symbiosis in leguminous plants involves physiological and biochemical properties of both the host plant and endosymbiont *(Brady) Rhizobium*. A precise exchange of molecular signals between the host plant and (brady)rhizobia over space and time is essential to the development of effective root nodules. The first apparent exchange of signals involves the secretion of phenolic compounds, flavones and isoflavones, by leguminous plants (Peters and Verma, 1990). These signal compounds are often excreted by the portion of the root with emerging root hairs, a region that is highly susceptible to infection by (brady)rhizobia (Verma, 1992). These compounds activate the expression of *nod* genes in (brady)rhizobia, stimulating production of the bacterial nod factor (Long, 1989; de Bruijn and Downie, 1991; Kondorosi, 1992). This nod factor has been identified as a lipo-oligosaccharide (Lerouge *et al.*, 1990), able to induce many of the early events in nodule development (Stacey *et al.*, 1992), including deformation and curling of plant root hairs, the initiation of cortical cell division, and induction of root nodule meristems (Dénarié and Roche, 1992). The isoflavones daidzein and genistein are the major components of soybean [*Glycine max* (L.) Merr.] root extracts responsible for inducing

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[1] To whom correspondence should be addressed: Fax: +1 514 398 7897. E-mail: ac10@musica.mcgill.ca

Abbreviations: DAP, days after planting; RZT, root zone temperature; PAL, phenylalanine ammonia lyase.

the *nod* genes of *Bradyrhizobium japonicum* (Kosslak *et al.*, 1987). Genistein has more *nod* gene-inducing ability than daidzein (Sutherland *et al.*, 1990).

Soybean is a subtropical legume that requires root zone temperatures (RZTs) in the 25–30 °C range for optimal symbiotic activity. Suboptimal RZTs (below 25 °C) are considered the major factor limiting soybean growth, nodulation and N_2 fixation (Whigham and Minor, 1978). However, infection and early nodule development processes are the most sensitive steps (Lynch and Smith, 1993; Zhang and Smith, 1994). Gibson (1971), in a review of the data regarding environmental effects on the legume–*Rhizobium* symbiosis, suggested that suboptimal RZTs retard root hair infection more than nodule initiation, nodule development or N assimilation. The time-course of each nodulation stage under optimal RZT conditions has been described by Turgeon and Bauer (1982). Bacterial attachment to root hairs occurs within minutes of inoculation and is followed, within 12 h, by marked curling of short root hairs. Infection threads, first visible within 24 h of inoculation, reach the base of the root hair by 48 h after inoculation. Under suboptimal RZTs, all of the infection steps were progressively delayed (Zhang and Smith, 1995). For example, the period between inoculation and root hair curling was 1 d and 2 d, respectively, for plants grown at 17.5 and 15 °C RZTs compared to 0.5 d for plants at 25 °C RZT. Studies on soybean N_2-fixation activity by fully formed nodules have also concluded that suboptimal RZTs directly decrease the activity of the nitrogenase enzyme complex (Layzell *et al.*, 1984).

Flavonoid levels have been shown to affect legume nodulation and N_2 fixation directly (Appelbaum, 1990). Cho and Harper (1991), for example, reported that a hypernodulating soybean mutant, derived from the cultivar Williams, had a higher root concentration of isoflavone compounds (genistein, daidzein, and coumestrol) than did Williams at 9–12 d after inoculation. Kapulnik *et al.* (1987) reported that the superior nodulation and N_2 fixation of HP32 alfalfa compared to HP alfalfa were associated with a 77% increase in the amount of plant tissue luteolin (a preferred inducer signal of *Rhizobium meliloti nod* genes). The effectiveness of isoflavones is found to vary between soybean cultivars (Horvath *et al.*, 1986; Zaat *et al.*, 1988). Matthews *et al.* (1989) showed that 3-d-old seedlings of the soybean cultivar Williams have a 10 times greater ability to induce *Bradyrhizobium nod* genes than do 3-d-old cultivar Bragg seedlings.

However, to date, there have been no investigations into the relationships between suboptimal RZTs and genistein concentration and content in plant root tissues. Recent studies showed that the preincubation of *B. japonicum* with genistein shortened the time between inoculation and infection thread initiation and increased soybean nodulation and N_2 fixation under controlled

environment conditions (Zhang and Smith, 1995). These results suggested that environmental factors probably altered the levels of genistein in the soybean root system. Therefore, the hypothesis that the genistein level of soybean roots will be altered when plants are exposed to suboptimal RZTs was tested in this study.

Materials and methods

Plant materials and growth conditions

Soybean (*Glycine max* L. Merr.) cultivar 'Maple Glen' was used throughout this study. This cultivar was selected as it has been developed for production under the short season, cool conditions of eastern Canada and it has performed well there. All of the experiments in this study were carried out on a controlled environment growth bench (Model GB48, Controlled Environments Ltd., Winnipeg, MB, Canada) at an irradiance of 300 μmol m^{-2} s^{-1} for a 16:8 h (day:night) photoperiod and a constant air temperature of 25 °C. There were ten plastic tanks (68 × 42 cm) in the growth chamber, and eight 13 cm plastic pots were sealed to the bottom of each tank. The RZTs were controlled (±0.5 °C) by circulating cooled water around the pots. A hole drilled in the tank bottom below each pot allowed the pots to drain when watered. A sterilized Turface (Applied Industrial Materials Corp., Deerfield, IL):sand (1:1, v/v) mixture was used as the rooting medium throughout this study. During each experiment plants were watered with a modified Hoagland's solution (Hoagland and Arnon, 1950), in which the $CaNO_3$ and KNO_3 were replaced with 1 mM $CaCl_2$, 1 mM K_2HPO_4 and 1 mM KH_2PO_4, to provide a nitrogen-free solution. Prior to each watering, the added Hoagland's solution was temperature-adjusted to the treatment RZT.

Experiment 1

Experiment 1 involved germination at six different RZT treatments, 13, 15, 17, 19, 22, and 25 °C and was arranged in a completely randomized design with four replications. Seed was surface-sterilized in sodium hypochlorite (3% solution containing 4 ml l^{-1} Tween 20), then rinsed several times with distilled water (Bhuvaneswari *et al.*, 1980). The seeds were directly sown into sterilized pots. When plants reached the vegetative-cotyledonary (VC) stage (unifoliolate leaves unrolled sufficiently that the edges were not touching [Fehr *et al.*, 1971]), plants were uprooted. Harvested roots were washed in distilled water and carefully dried using paper towels, prior to extraction of genistein.

Experiment 2

Experiment 2 involved transfer of germinated seedlings into different RZTs and used the same experimental design as experiment 1, a completely randomized design with four replications. Six RZTs, 13, 15, 17, 19, 22, and 25 °C, were also included in this experiment. The sterilized seeds were first planted in trays at 25 °C. Ten-day-old seedlings at the VC stage were transplanted into each pot. After 2 d of acclimatization, plant roots were harvested for genistein extraction (same process as in experiment 1).

Experiment 3

This experiment involved time-course harvests of plants germinated at different RZTs and was arranged in a split-plot design

with four replications. The main-plot unit was RZT at four levels, at 25 °C (optimal temperature for soybean nodulation and N$_2$ fixation [Jones and Tisdale, 1921; Dart and Day, 1971]), 19 °C (sub-optimal temperature but still above the critical 17 °C, below which soybean nodulation and N$_2$ fixation were strongly inhibited [Lynch and Smith, 1993; Zhang et al., 1995]), 17.5 °C (half a degree above critical point [17 °C] which strongly inhibited soybean nodulation and N$_2$ fixation), 13 °C (below the critical point at which soybean nodulation was strongly inhibited [Lynch and Smith, 1993; Zhang and Smith, 1994]). The sub-plot unit consisted of different harvest stages. In this experiment sterilized seeds were directly sown into the four RZTs. When the plants grown at 25 °C RZT reached the vegetative 1 (V1) stage (12 DAP), they were uprooted and the root systems were washed in distilled water, paper towel-dried and then used to determine root genistein concentration. After the first harvest, plant roots were harvested at 3 d intervals, to a total of six harvests. At 19 °C RZT, the first harvest was conducted at the same time as the second harvest of plants grown at 25 °C RZT; plant roots were then harvested every 3 d to a total of six harvests. The same procedure was followed for all other main-plot units (Table 1), with the single exception that for plants grown at 13 °C there were only five harvests. These plants produced less root material, so that only five harvests could be carried out.

Experiment 4

Experiment 4 was similar to experiment 3, but involved transplanted seedlings as for experiment 2. It had the same experimental design, including main-plot and sub-plot units, as experiment 3. In this experiment sterilized seeds were planted in trays at 25 °C. Ten day-old seedlings at the VC stage were transplanted from these trays into pots at a range of RZTs (13, 17.5, 19, and 25 °C). The first harvest of plant roots for analysis was at the time of transplanting, followed by five additional harvests at 3 d intervals (Table 2).

Plant inoculation

Because the plants of experiments 1 and 2 were harvested after only 2–10 d of exposure to treatment RZTs, prior to significant nodule development at most RZTs, plants used in those experiments were not inoculated. Inoculum was supplied to the plant roots in both experiments 3 and 4 at 10 DAP. The inoculum was produced by culturing *B. japonicum* strain 532C (Hume and Shelp, 1990) in yeast extract mannitol broth (Vincent, 1970) in 250 ml flasks shaken at 125 rpm at room temperature (23 ± 2 °C). Strain 532C has been shown to perform well over a range of temperatures (Lynch and Smith, 1993). When the subculture reached log phase, distilled water was used to dilute the inoculum to an A_{620} of 0.08 (approximately

Table 2 *Description of plant development stage (Fehr et al., 1971) at different harvests over four different root zone temperatures (data from experiment 4)*

RZT	DAP				
	13	16	19	22	25
25 °C	1/3 V2[a]	V2	1/2 V3[b]	V3[c]	V4[d]
19 °C	1/3 V2	V2	1/2 V3	V3[b]	V4[c]
17 °C	1/3 V2	V2	1/2 V3	V3	V4[b]
13 °C	1/3 V2	V2	1/2 V3	V3	V4

[a]1/3 V2 means the plants were a third of the way to reaching the V2 stage, e.g. the first trifoliate leaf was expanded a third; same for all others.
[b]Nodules were visible at this harvest, but the diameter of the nodules was less than 1 mm.
[c]Nodules were visible and the diameter of the nodules was greater than 1 mm.
[d]The colour of the nodules becoming pink, the nodules seemed to be functioning.

10^8 cells ml^{-1}) (Bhuvaneswari et al., 1980). The inoculum was cooled to the corresponding RZT and 1 ml was applied by pipette on to the rooting medium at the base of the plant.

Preparation of plant extracts for high pressure liquid chromatography (HPLC) analysis

Harvested soybean roots were placed in vacuum flasks with ten times the root fresh weight of 80% methanol-dH$_2$O. Root tissue was put under vacuum for 15–20 min, and then the pH was adjusted to 5.3 with 0.1 N HCl. Samples were transferred into 250 ml flasks at 4 °C and shaken for 48 h at 150 rpm. Plant roots and extracts were separated by Whatman No. 1 filter paper. Plant roots were dried at 70 °C over 48 h and weighed. Plant root extracts were rotary evaporated to remove the methanol fraction. The aqueous fraction was freeze-dried, and resuspended to equal 10 mg extract dry weight per ml of distilled water. The resuspended extracts were phase-partitioned three times against equal volumes of ethyl acetate, retaining each organic fraction. Dry sodium sulphate was added to absorb the remaining water. The clear ethyl acetate fraction was decanted and rotary evaporated at 30 °C until dry. The sample was then redissolved in 1 ml of HPLC grade pure methanol and centrifuged for 15 min before use. The genistein concentration was determined using a Waters HPLC system (Waters Associates Inc., Milford, MA) consisting of a model 712 WISP, two 510 pumps and a 441 UV detector operating at 254 nm. Separation was achieved by a 3.9 × 300 mm μBondapak C18 column (Waters Associates Inc. Milford, MA) using a mobile phase consisting of a 60:40 ratio of methanol:distilled water. The

Table 1. *Description of plant development stage (Fehr et al., 1971) at different harvests over four different root zone temperatures (data from experiment 3)*

RZT	DAP							
	12	15	18	21	24	27	30	33
25 °C	V1	1/2 V2[a]	1/2 V3	1/2 V3[b]	2/3 V4[c]	1/3 V5[d]		
19 °C		V1+	V2	1/3 V3	1/3 V4[b]	2/3 V4[c]	V4[d]	
17 °C			V2	1/3 V3	V3	1/3 V4[b]	1/2 V4[b]	V4[c]
13 °C				2/3 V2	V2	1/3 V3	1/2 V3	V3

[a]1/2 V2 means the plants were half-way to reaching V2 stage, e.g. the first trifoliate leaf was expanded half way; same for all others.
[b]Nodules were visible at this harvest, but the diameter of the nodule was less than 1 mm.
[c]Nodules were visible and the diameter of the nodule was greater than 1 mm.
[d]The colour of the nodules becoming pink, the nodules seemed to be functioning.

run time for separation was 15 min with an isocratic flow rate of 1.0 ml min^{-1}. HPLC chromatograms of the genistein from root extracts of plants grown at different RZTs were identified by use of a commercial genistein standard (4', 5, 7-trihydroxyisoflavone, purity of 98%, from Sigma) (Fig. 1).

Statistical analysis

Results were analysed statistically by analysis of variance using the Statistical Analysis System computer package (SAS Institute Inc., 1988). When analysis of variance showed significant treatment effects, the LSD test was applied to make comparisons among the means at the 0.05 level of significance (Steel and Torrie, 1980).

Results

HPLC analysis

Extracted genistein was identified in the HPLC eluate by retention time. The genistein peak eluted at the same time and had the same spectrum as the commercially obtained genistein with a peak retention time of approximately 8.3 min (Fig. 1).

Experiment 1

Root genistein concentration of plants maintained at 25 °C RZT was 0.75 μg g^{-1} of fresh weight, which was not different from that at the RZTs of 22 °C and 19 °C (Fig. 2A). However, for plants germinated either at, or below 17 °C RZT, root genistein concentrations were lower than those of plants with roots maintained above 17 °C. Root genistein concentrations were not different among RZTs of 13, 15 and 17 °C (Fig. 2A). Genistein content per root system generally followed the same pattern as the genistein concentration (Fig. 2B), except that plants maintained at 13 °C RZT had lower root
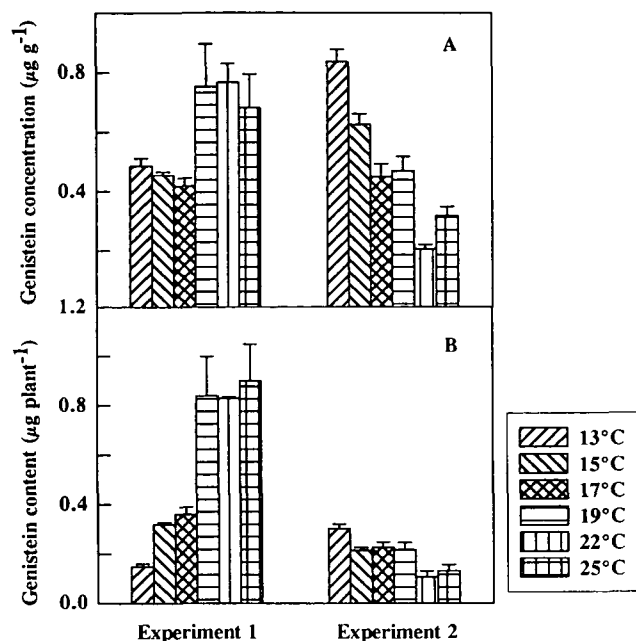

Fig. 2. Genistein concentration (μg g^{-1}) (A) and content (μg plant^{-1}) (B) of soybean roots exposed to 13, 15, 17, 19, 22, and 25 °C RZTs (experiments 1 and 2). In experiment 1, the soybean plants were directly sown in different RZTs, whereas in experiment 2, plants were germinated at the optimal 25 °C RZT, then transferred into different RZTs. Each bar represents the mean ± 1 SE ($n = 20$).

genistein contents than those of plants at 15 °C and 17 °C RZTs.

Experiment 2

The root genistein concentration responses to RZT in this transfer experiment were the reverse of those in the
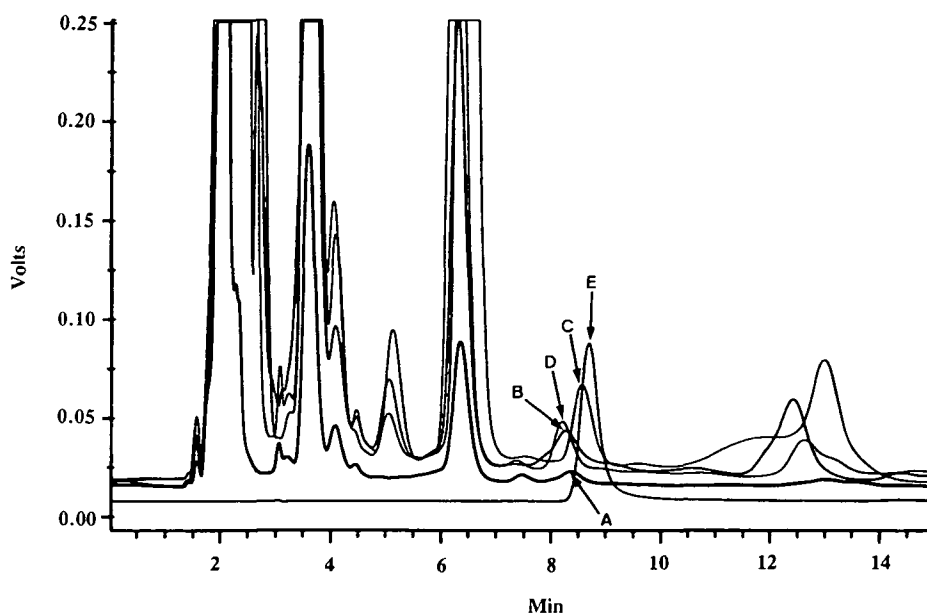

Fig. 1. Comparison of high pressure liquid chromatography (HPLC) chromatograms. Chromatograms of root extracts at 24 d after planting in experiment 3. Plant root systems from (A) 15 °C, (B) 17.5 °C, (C) 19 °C, and (D) 25 °C, and (E) for genistein standard at 50 μM.

first experiment. Plant roots that were initially grown at 25 °C RZT then acclimated at the lower RZTs for 2 d had higher genistein concentrations and contents (Fig. 2) than plants with roots maintained at 25 °C or transferred from 25 °C to 22 °C RZT. Genistein concentration appeared to decrease linearly with increasing acclimation RZT (Fig. 2A). Genistein content for roots acclimated at different RZTs followed the same pattern as plant root genistein concentration. Roots acclimated at 13 °C RZT had the highest genistein content; the genistein content declined as the acclimation RZT increased (Fig. 2B).

Experiment 3

Averaged over all harvests, genistein concentrations of soybean roots exposed to different RZTs had the same general pattern as experiment 1 where only one harvest was made at the VC stage. The root genistein concentrations of plants grown under RZTs of 17.5, 19 and 25 °C (above 17 °C) were 1.57, 1.71 and 1.17 μg g^{-1}, respectively, which were higher (LSD$_{0.05}$=0.20) than the 0.94 μg g^{-1} of roots at 13 °C RZT (below 17 °C). The genistein content per root system also followed the same pattern as in experiment 1. Genistein contents per root system for plants maintained at 17.5, 19 and 25 °C RZTs were 4.40, 5.16 and 3.12 μg, respectively, higher than the 0.61 μg of plant roots at 13 °C RZT (LSD$_{0.05}$=1.89).

Over the different harvest stages the genistein concentration of plant roots at 25 °C RZT increased before the onset of N$_2$ fixation. When the plant nodules began fixing nitrogen (24 DAP [14 d after inoculation]), root genistein concentration declined (Fig. 3A). The genistein concentration curve for plant roots at 19 °C and 17.5 °C showed the same pattern as that of plants with roots maintained at 25 °C RZT; however, the genistein concentration increased more dramatically for plant roots maintained at 19 °C and 17.5 °C RZTs than at 25 °C RZT. Following the 21 DAP harvest, the genistein concentrations of plant roots at 17.5 °C and 19 °C were higher than those of plant roots maintained at 25 °C RZT. The genistein concentration of plant roots maintained at 13 °C RZT was always the lowest. The relationship between RZTs and genistein content per plant root system across different harvests followed the same pattern as genistein concentration. Before the onset of N$_2$ fixation, the genistein content of plant roots maintained at 25 °C and 19 °C RZTs followed the same pattern; however, the genistein content peak for 25 °C and 19 °C RZTs appeared at 24 and 27 DAP (just before nodule colour change, Table 1), respectively. The genistein content of plant roots maintained at 17.5 °C was lower than those of plant roots at 25 and 19 °C RZTs before plants grown at 25 and 19 °C started fixing N$_2$. For the plants with roots maintained at 13 °C RZT, the root genistein content was lower than for root systems at the other three RZTs, regardless of whether the compar-
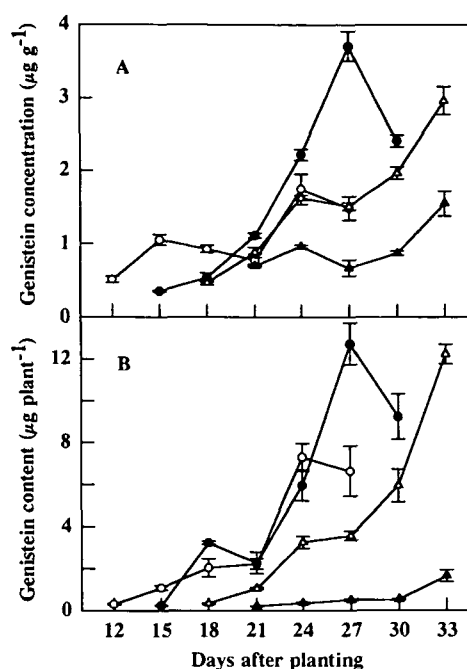

Fig. 3. Root genistein concentration (A) and content (B) curves of soybean plants maintained at 13 °C (▲), 17.5 °C (△), 19 °C (●), or 25 °C (○) during early vegetative growth (experiment 3). The data represented are from experiment 3. Each value is plotted as the mean ± 1 SE (n = 20).

isons were made based on harvest date or plant growth stage (Fig. 3B).

Experiment 4

Results from this experiment supported those of experiment 2 in that the root genistein concentrations of plants maintained at 25 °C or transferred from 25 °C to 19 °C were less than those transferred to lower RZTs (17.5 °C and 13 °C) at the second harvest which most closely approximates the single harvest of experiment 2. Averaged over the five harvests after transplanting, root genistein concentrations of plants exposed to 13, 17.5, 19, and 25 °C RZTs were 1.53, 1.42, 1.49, and 1.31 μg g^{-1}, respectively, and were not different from each other (LSD$_{0.05}$=0.26). Averaged over the five harvests after transplanting, root genistein content per plant had the same pattern as experiments 1 and 3, i.e. plant root genistein content increased with increasing plant RZTs; the contents were 3.03, 3.59, 5.10, and 5.70 μg per plant for plants acclimated at 13, 17.5, 19, and 25 °C RZT (LSD$_{0.05}$=1.44), respectively.

Although genistein concentration was the same for all plant roots at the first harvest (time of transplanting), it began to differ among RZTs after only 3 d of acclimation. At 13 DAP, plant roots at 17.5 °C had the highest genistein concentration level, while after 19 DAP plant roots at 13 °C RZT had the highest level, and they continued to do so until 25 DAP. Plants acclimated at

25 and 22 °C RZTs had the lowest genistein concentrations before 19 DAP; this then increased and finally surpassed that of plants at 17.5 °C RZT following the 19 DAP harvest. By the last harvest, it appeared that the concentrations in the lower RZTs (13 °C) were beginning to decrease relative to the higher RZTs (19 and 13 °C had crossed over), except for the 25 °C RZT treatment, which began to fix nitrogen 1 d before the final harvest (14 d after inoculation) (Table 2; Fig. 4A). At the 13 DAP harvest plant roots acclimated at both 25 and 17.5 °C had the highest genistein contents, whereas the plant roots acclimated at RZTs of 19 °C or 13 °C had the lowest. After 6 d of acclimation, plant roots maintained at 25 °C or acclimated at 19 °C had higher genistein contents per plant root (Fig. 4B). At the last harvest, plant roots at 19 and 25 °C RZTs had double the genistein content of plant roots at 13 and 17.5 °C RZTs.

Discussion

The optimal temperature range for soybean growth and development is 25–30 °C, temperatures which fall outside the optimal range cause stress (Jones and Tisdale, 1921). The primary effect of low temperatures on plants is the reduction in rate of growth and metabolic processes. Isoflavones have been shown to be prominent compounds in chickpea and soybean tissues (Barz and Welle, 1992). Cicer arietinum plants and cell cultures accumulate mainly formononeitin and biochanin A, whereas soybean tissues

Fig. 4. Root genistein concentration (A) and content (B) curves of soybean plants transferred (at 10 d after planting) from 25 °C to 13 °C (▲), 17.5 °C (△), or 19 °C (●), or maintained at 25 °C (○) during early vegetative growth (experiment 4). The data represented are from experiment 4. Each value is plotted as the mean ± 1 SE (n = 20).

are rich in daidzein and genistein. In both systems these isoflavones predominantly occur in the form of 7-O-glucoside 6″-O-malonate conjugates (Köster et al., 1983; Graham et al., 1990). The isoflavone conjugates are located in vacuoles, as recently demonstrated with chickpea cells (Mackenbrock et al., 1992). Eleven enzymes, including phenylalanine ammonia lyase (PAL), and chalcone isomerase, are involved in the biosynthesis of genistein in soybean plants (Barz and Welle, 1992). The characteristic spectrum of proteins produced by a particular plant organ is often altered at temperatures close to or below the limit for optimal growth (Graham and Patterson, 1982). In general, most enzymes are more stable under low temperature conditions although their activity usually decreases as temperature decreases and their concentration may change. For instance, PAL catalyses the first step in the synthesis of compounds such as cinnamic acid from phenylalanine, which are steps in the pathway leading to the biosynthesis of genistein. For many plants the extractable amount of PAL increases when the whole plant or detached parts are acclimated to low temperature conditions (Graham and Patterson, 1982). PAL is turned over rapidly at normal temperatures (Glasziou, 1969), and it seems likely that the level of PAL increases at low temperature because the rate of synthesis is decreased less by cold than is the rate of degradation (Graham and Petterson, 1982). The biosynthesis of genistein in plant tissues may be reduced under low temperature conditions by declining relative activity of enzymes in the synthesis pathway. These studies showed that the roots of plants germinated and grown at lower RZTs had lower genistein concentrations and contents than plants grown at higher RZTs (Figs 2, 3).

When the plants were transferred from an optimal RZT to lower RZTs, genistein concentration of root tissues increased compared to the plants maintained at 25 °C RZT, or transferred to 22 and 19 °C RZTs in the first few days (Fig. 2 or 4). After 7–10 d of acclimation, the genistein concentrations of plants maintain at 25 °C, or acclimated at 19 °C RZT were higher than those of plants acclimated at 17.5 and 15 °C RZTs in experiment 4 (Fig. 4). There are several possible explanations for the transient increase in root genistein concentration in the first few days, and decrease thereafter. The first possible reason could be a reduction in the excretion of genistein from root cells into the rhizosphere, with the rate of genistein excretion being reduced by low temperature more than the rate of its biosynthesis. At the membrane level, when plants cells or their detached parts are first exposed to low temperature conditions, membrane fluidity, and thus function, are reduced. In order to retain a reasonable level of membrane fluidity the cell membrane unsaturated fatty acid concentrations increase (Devlin and Witham, 1983), with increasing cell acclimation time under low temperature conditions (Willemot, 1975).
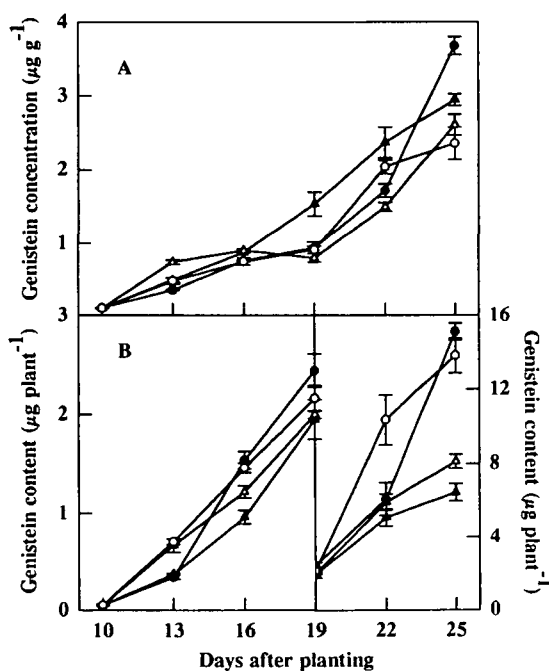
Therefore, it could be that membrane fluidity was initially reduced by suboptimal RZTs, and this led to reduced rates of genistein excretion, relative to synthesis, and that after a period of adaptation and change in membrane lipid composition, fluidity was restored and excretion rate increased relative to synthesis.

Second, as described above the biosynthesis of genistein in soybean plants includes eleven enzymes (Barz and Welle, 1992); it may be that some enzymes involved in the synthesis of genistein are more sensitive to low RZT, and that prior to acclimation of plants to low temperatures, those enzymes have been synthesized. Once the plants are transferred to suboptimal RZTs both genistein synthesis and excretion are inhibited, but metabolism of the various intermediates, already accumulated, goes ahead and more genistein is formed for a while. However, after these pools of genistein precursors are exhausted, genistein concentration at lower RZTs again become lower than those at higher RZT (Fig. 4). In a similar fashion, root chilling may promote the conjugation of genistein or inhibit the conversion of conjugated into free genistein. Genistein has been found to exist as free aglycones and as multiple glycosyl conjugates in soybean plants. The conjugates have been identified as 7-*O*-*β*-D-glucosyl-genistein and the corresponding 6″-*O*-malonyl conjugates. In *vitro*, 7-*O*-*β*-D-glucosyl-genistein hydrolysed under strong acid (1 N HCl, 100 °C, 1 h) or *β*-glucosidase to free genistein (Graham *et al.*, 1990). In *vivo* these conjugates are assumed to act as a reservoir of genistein that can readily be mobilized if the need arises (Barz and Welle, 1992). Any change which caused an increase in genistein conjugation rate, a decrease in the rate of genistein liberation from conjugates or both, would change the concentration of free genistein in roots. It is the free genistein that is the active form.

Third, the relative genistein concentration increased by reduction in water content of plant root cells. When plant cells are exposed to low temperatures, they usually lose some water as a method of protecting themselves from chilling injury (Wilson and McMurdo, 1981). These experimental results, at 3 d after transfer (second harvest), showed that the ratios of root fresh weight and dry weight were 27.7, 26.1, 24.2, and 25.0 for plants maintained at 25 °C, or acclimated at 19, 17.5, and 13 °C RZTs, respectively. At the third harvest (6 d after transfer) the difference of these ratios between RZTs were 25.0, 26.6, 23.4, and 20.3 for plants at 25, 19, 17.5, and 13 °C, respectively. Afterwards, the changes in water content among different temperatures were too small to contribute to the changes in genistein concentration and, after the third harvest, the differences of these ratios were no longer present (data not shown).

Based on genistein concentration and content in experiment 1, RZTs can be divided into two groups, 19–25 °C, and 13–17 °C. The genistein concentrations of plants grown at RZTs within either one of the two groups were not different (Fig. 2A). The genistein content per plant root system followed the same pattern as the genistein concentration in soybean roots, except that plants germinated at 13 °C had the lowest level compared to the other five RZTs (Fig. 2B). This was because of much smaller roots for plants at 13 °C RZT. These results agreed with our previous findings that 17 °C is a threshold RZT for soybean nodulation; when soybean plants grow between 25 and 17 °C RZT the time for nodule development (between inoculation and onset of nitrogen fixation) was increased by 2–3 d for each degree decrease in temperature, while below 17 °C each degree appears to increase the interval by about 7 d (Zhang *et al.*, 1995).

In summary, these experiments showed that (1) when soybean plants were maintained at RZTs ranging from 13 to 17 °C, genistein concentrations and contents per plant were lower than those of plants maintained at RZTs above 17 °C; (2) when plants were transferred from the optimal RZT to RZTs below 17 °C, then acclimated for a few days, genistein concentrations and contents were transiently higher than those of plants either maintained at optimal RZT, or acclimated at a RZT above 17 °C; (3) under constant temperature conditions genistein concentration increased before the onset of nitrogen fixation, and decreased sharply thereafter; (4) part of the effect of RZTs on genistein content per plant was due to the changing genistein concentration at different RZTs, while part was due to low RZT decreasing the plant root growth. Overall, low RZTs reduced genistein accumulation in soybean roots. Although direct effects on genistein excretion from soybean roots remains to be demonstrated, this work shows clearly that the pattern of temperature effect on soybean nodulation is repeated for soybean root genistein concentration.

Acknowledgements

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