

**THE SYMBIOTIC INTERACTION OF
BRADYRHIZOBIUM JAPONICUM WITH BAMBARA
GROUNDNUT AND COWPEA AND THE EFFECTS OF
NOD GENE-INDUCERS, DAIDZEIN AND GENISTEIN.**

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INTRODUCTION

Legumes are an interesting group of plants in that they have their own nitrogen supply which is a considerable advantage, particularly in the nutrient poor soils of Africa (FAO). Bacteria of the genera *Rhizobium*, *Azorhizobium* and *Bradyrhizobium* infect leguminous plants and establish a nitrogen fixing symbiosis in a unique plant organ, the nodule (Gresshoff, 1992). This interaction involves physiological and biochemical processes of great significance to both agriculture and natural ecosystems (Pueppke *et al.*, 1998). The process of nodulation initially involves the selective attachment to and internalisation of the plant root by the bacterium, once mutual recognition has occurred (Zhang and Smith, 1995).

The process of recognition between symbionts involves a precise exchange of low-molecular weight signal molecules between rhizobia and the host plant over space and time and, is critical if effective root nodules are to be formed (Verma, 1992). The release of flavonoids from the roots with emerging root hairs initiate a series of bi-directional molecular signals which results in the nitrogen-fixing symbiosis (Rao and Cooper, 1995; Redmond *et al.*, 1986). The initial steps in the plant-microbe interaction essentially involves the legume roots secreting specific flavonoids or isoflavonoids which are recognised by the microsymbiont (deBruijn and Downie, 1991). Subsequently nodulation genes (*nod/nol*) are induced in the microsymbiont, this effect causes the

microsymbiont to release host-specific lipooligosaccharide reverse signal molecules (Nod factor) which causes hair curling and cortical cell division in plant roots leading to nodule formation (Gottfert, 1993). The gene product of the regulatory nodD gene, is dependant on the plant signals to convert it to an active form (Rolfe, 1988). The altered NodD protein then induces the expression of other nodulation genes (Rolfe, 1988). Increased concentrations of these plant signals result in greater transcription of nodulation (nod) genes and therefore greater nodule formation (Dakora and Nyamekye, 1997). The bacterium , once induced by the plant signals is only then able to infect the host root hairs (Rolfe, 1998). Most symbiotic associations are fairly specific, however some bacterial strains of rhizobia and legume species are non-specific in their choice of symbiotic partners (Puekke *et al.*, 1998). Several studies have demonstrated that different legumes can use the same molecules to signal to their different homologous microsymbiont (Dakora, Joseph and Phillips, 1993; Hungria, Joseph and Phillips, 1991; Dakora and LeRoux). ref⁹

Molecular signals between legume roots and rhizobia in the rhizosphere determines the specificity, most of which have been identified to be isoflavonoids (Phillips *et al.*, 1991). The development of an assay system has led to the characterisation of the specific compounds responsible for nod gene activation in rhizobia: from clovers, 7,4'-dihydroxyflavone; from alfalfa, luteolin; from peas, apigenin; and from soybeans, the isoflavones daidzein and genistein (Rolfe, 1988). These hydroxyl bearing flavonoid compounds are

synthesised in the phenylpropanoid pathways also known for the synthesis of many important plant phenolic compounds such as phytoalexins involved in plant defence strategy (Rolfe, 1988). Isoflavonoids (subclass of flavonoids) including genistein, daidzein, and the coumestran, coumestrol; have been shown to induce resistance in *Bradyrhizobium japonicum* and *Rhizobium fredii* to the phytoalexin, glyceollin (Parniske *et al.*, 1991). Krishnan and Pueppke (1993) have identified several new proteins following preincubation of *R. fredii* with genistein at the nod gene-concentrate which indicates that isoflavonoids could influence the expression of genes other than those directly involved in Nod factor synthesis (Rao and Cooper, 1995).

The purpose of this kind of research is to further investigate the potential of flavonoids or isoflavonoids. These compounds have been studied for their agronomic importance because when detected in plants they deter insects and pathogens of agronomically important crop plants (Dakora, 1995). These compounds also elicit flavour to food, provide the attractive colours to flowering plants thus influencing reproductive potential (Dakora, 1995).

The aim of this project was to investigate whether nodulation, and nitrogen concentration of legumes can be increased by providing additional nod gene-inducer compounds. Both daidzein and genistein are nod gene-inducers for rhizobia nodulating cowpea, bambara groundnut, soybean and the common

bean. The question is: Which one of them show greater ability to promote nodule mass? the data from one experiment involving cowpea and bambara groundnut will indicate whether (i) adding nod gene-inducers promote nodule formation and nitrogen content, and, (ii) which of the two, daidzein or genistein, has greater efficacy in eliciting nodule formation.

The data from a second experiment involving pre-incubation of nod gene-inducers with *Bradyrhizobium japonicum* strain CB 756 will indicate whether adding these signal compounds to bacterial inoculants will promote greater nodulation and nitrogen fixation.

MATERIALS AND METHODS

Experimental design.

The experiments were carried out in a glasshouse at the University of Cape Town. Temperature and light were not controlled and therefore imitated field conditions.

Two sets of experiments were performed:

Experiment 1

The seeds of cowpea and Bambara groundnut were planted on the 1st of December in 3 litre pots filled with 1.5 kg sand. Cowpea germination proceeded on the 7th of December while Bambara groundnut germinated on the 12 of December.

Experiment 1 consisted of 7 treatments each with 4 replicates. The treatment commenced from the 14th of December. until the 18th of December until all seedlings were inoculated with *Bradyrhizobium* strain CB 756. Each pot received 300ml of deionised water per day. The treatments were applied for 5 consecutive days, and involved giving each plant 5 ml of the following treatments:

daidzein

0uM

10uM

50uM

genistein

0uM

10uM

50uM

daidzein+ gensitein

10uM+10uM

50uM+50uM

Experiment 2

Plants of both cowpea and bambara groundnut were raised under nitrogen free conditions in Leonard jars (Dahiya & Khurana, 1981) comprising 5 treatments each with 4 replicates. Cowpea germination proceeded on the 11th of December while Bambara germination began on the 15th of December. Seedlings were inoculated with broth culture containing daidzein and genistein respectively (350 ml each). Treatments commenced 40 days after sowing (DAS).

The treatments were 5 ml/day as follows:

Uninoculated

Inoculated

Inoculant + 50uM daidzein

Inoculant+50uM genistein

Inoculant+ (50 uM daidzein+50uM genistein)

Surface sterilisation of seeds

Clean seeds of cowpea (*Vigna unguiculata*) and Bambara groundnut (*Vigna subterranea* L. Thouars) were surfaced sterilised. Seeds were placed in Erlenmeyer flask (wide-mouthed and previously sterilised by autoclaving). The flask was covered with a sterile petri dish half. The seeds were rinsed in 95% alcohol (EtOH) for 10 seconds and drained off. 50 ml of 50% home bleach solution was added to immerse seeds. Sterilant and seeds were mixed, gently after 60 seconds; the sterilant was drained off. The seeds were then rinsed ten times with sterile distilled water.

Preparation of Leonard Jars

Modified Leonard jars were assembled consisting of 700 ml capacity beer bottle with the lower portion cut off. The beer bottle was inverted into a heavy 2-litre glass jar. The mouth of the bottle was situated 3cm above the base of the glass jar. The growth medium (sand) in the bottle was irrigated by a centrally positioned cotton wick of 45m, 10cm extending out of the mouth of the beer bottle. Small amounts of absorbent cotton were stuffed into the neck of the bottle. Enough growth medium (sand) was used to fill the beer bottle with

wick in place. Seeds of Bambara and cowpea was planted after which the bottle was positioned in the reservoir. Pouring 100 ml of Hoaglands N-free nutrient solution (Hewitt, 1966) moistened the sand. The glass jar was filled with 1600 ml of Hoaglands N-free nutrient solution. The whole apparatus was covered with aluminium foil and secured with tape. One half of glass petri dishes halves were used as caps to cover the beer bottles. The complete assembly and nutrient solution was sterilised by autoclaving for 3 hours at 121°C and 15 psi and left to cool overnight. Once cooled the Leonard jars were transferred to the laminar flow hood and sown with sterilise seeds of each legume species and, transferred to the greenhouse.

Preparation of nutrient solution.

The modified Hoaglands solution (Hewitt, 1966) was prepared in thoroughly cleaned 20 litre containers. Before application of nutrient solutions, containers were shaken each time to homogenise the contents. The preparation was performed according to the outline in Table 1.

Preparation of Genistein and Daidzein concentrations

50uM Daidzein

0.0254g daidzein was dissolved in 50 ml of 0.1M NaOH and made up to 500ml with water.

10 μ M Daidzein

400 ml of 50 μ M stock solution was diluted to 2 litres to give a 10 μ M daizein solution.

50 μ M Genistein.

0.027025 g genistein was dissolved in 150 ml of 80% ethanol and made up to 500ml with water.

10 μ M Genistein

400 ml of 50 μ M stock solution was diluted to 2 litres to give a 10 μ M genistein solution.

Yeast Mannitol Broth

The broth was prepared according to Table 2. A total volume of 600 ml was prepared and autoclaved for approximately 3 hours at 121°C and 15 psi. Each

container received 300 ml of yeast mannitol broth prepared each for preincubation with genistein and daidzein.

Broth culture preparation

20 ml of 50 μ M genistein was added to yeast mannitol broth and homogenised. The agar slant containing *Bradyrhizobium* strain CB 756 received 2.5 ml of sterilised water. Slant and sterile water were mixed thoroughly. 2 ml of liquid agar was added to the sterile broth culture. The broth culture with added nod gene-inducer was placed in a rotar shaker (50 rpm) at 28°C for 36 hours.

Total N determination.

Harvesting and Sample Preparation.

Cowpea and bambara groundnut of both experiments were harvested on 13th of January. Three weeks after the commencement of the above mentioned treatments. The plants were separated into shoots (stems and leaves), roots and nodule fractions and dried at 50°C for 48 hours. The samples were then weighed and ground to a 40 μ m size and transferred to plastic vials

Total Nitrogen

Total N-analysis was performed at Infruitec Soils Science department. About 250 mg shoot and 150 mg root material were weighed into Kjeldahl digestion tubes. The total nitrogen in each sample was converted into ammonium sulphate by Kjeldahl digestion using selenium catalyst tablets and 10 ml concentrated sulphuric acid and three glass beads. Cold digestion took 15 hours.

Statistical Analysis

Analysis of variance (ANOVA) and descriptive statistics were used to reveal significant differences among treatments per experiment per plant.. Post hoc comparison was performed. LSD test were used to test for significance among means per treatment.

Table 1. Modified Hoagland Solution (Hewitt, 1966)

| Reagents | molecular weight | stock solution on (g/l) | 1/3full strength |
|---|------------------|----------------------------|------------------|
| MgSO ₄ . 7H ₂ O (1M) | 246.48 | 246.48 | 21 |
| CaCl ₂ (1M) | 110.99 | 111.0 | 21 |
| K ₂ SO ₄ (0.5M) | 174.27 | 87.14 | 21 |
| KH ₂ PO ₄ (1M) | 136.09 | 68.0 | 10 |
| K ₂ HPO ₄ (1M) | 174.18 | 87.1 | 10 |
| Sequestrene (138 Fe) Fe chelate | | 18.7 | 43 |
| MnCl ₂ . 4H ₂ O | 197.91 | .0724 | 10 |
| ZnCl ₂ | 136.28 | .11 | 10 |
| CuCl ₂ .2H ₂ O | 170.48 | 0.07 | 10 |
| Na ₂ MoO ₄ . 2H ₂ O | 241.05 | 0.025 | 10 |
| CoCl ₂ . 6H ₂ O | 237.95 | 0.06 | 10 |
| H ₃ BO ₃ | 61.83 | 5.72 | 10 |

Table 2. Yeast Mannitol Broth

| | |
|-------------------------|----------|
| K_2HPO_4 | 0.125 g |
| $MgSO_4 \cdot 7H_2O$ | 0.05 g |
| NaCl | 0.025 g |
| Mannitol ⁽¹⁾ | 2.5 g |
| Yeast ⁽²⁾ | 0.4 g/ L |
| Distilled water | 225 ml |

RESULTS

Dry matter accumulation

Experiment 1

Bambara groundnut

Overall plant growth

Overall plant growth expressed as dry matter accumulation was significantly different for treatments. Treatments 3, 4, 7 showed a significant ($p \leq 0.05$) decrease in total dry matter. Treatment 2 showed a 17% increase in dry matter accumulation although not statistically significant ($p > 0.05$) (fig. 1d).

Shoot dry matter

The shoots accumulated significantly ($p \leq 0.05$) greater dry matter than either the roots or the nodules for all treatments. There were significant ($p \leq 0.05$) differences between treatments. However the proportion of shoot dry mass to whole plant dry mass was almost all similar (fig. 1a).

Root dry matter

Similarly, the proportion of root dry matter to whole plant dry matter was almost similar for all treatments. A significant ($p \leq 0.05$) difference between treatment were observed. There was a significant ($p \leq 0.05$) increase in root dry matter for treatment 2, (25%) 3, (35%) and 5 (29%) (fig. 1b).

Nodule dry matter

There were significant ($p \leq 0.05$) differences between treatments. Treatment 2 there was a significant ($p \leq 0.05$) decrease in nodulation compared to control. There was a 12% decrease in nodule dry matter at treatment 3 although not statistically significant ($p > 0.05$) (fig 1c).

Cowpea

Overall plant growth

Dry matter accumulation was significantly ($p \leq 0.05$) different between treatments. Treatment 7 showed a significant ($p \leq 0.05$) decrease in total dry matter relative to treatments 2, 3, 4. These latter treatments showed an increase in total dry matter compared to control, 35%, 29% and 16% respectively although not significant ($p > 0.05$) (fig 2d).

Shoots

Statistically there was no significant ($p > 0.05$) differences between treatments. Shoot dry matter was significantly lower than root dry matter. At treatments 2, 4, and 6 a significant ($p \leq 0.05$) increase in dry matter was observed. At treatments 3, and 7 there was a significant ($p \leq 0.05$) decrease. Note that the proportion of root dry matter to whole plant dry matter was not similar (fig 2a).

Nodule dry matter

Nodule dry matter showed a significant ($p \leq 0.05$) increase at treatment 2, 3, and 5. There was no proportional similarity of root dry mass to whole plant dry matter. There were no proportional similarity between nodule dry mass to total dry mass/ plant (fig 2c).

Experiment 2

Bambara groundnut

Overall plant growth

Upon harvesting visible differences were observed for treated plants. Discoloration of roots for treatment 3 and 4 were observed. Total dry mass accumulation was significantly ($p \leq 0.05$) different among treatments. There was a significant ($p \leq 0.05$) increase in total dry mass for treatment 3 compared to treatments 1 and 2, 82% relative to control was observed (fig 3d).

Shoot dry matter

The proportion of shoot dry matter to whole plant dry matter was not similar. Shoot dry matter accumulation is significantly ($p \leq 0.05$) different among treatments. Treatment 2 and 4 showed significant increases in dry matter relative to control. Treatments 3 and 5 showed no significant difference from

control. Treatment 4 shows the highest significant ($p \leq 0.05$) increase relative to control at 21%(fig 3a).

Root dry matter

There were significant ($p \leq 0.05$) differences among treatments. Treatments 2, 3 and 4 showed a significant ($p \leq 0.05$) increase in dry matter relative to control. A steady increase was observed from treatments 2 to 4 (fig 3b).

Nodule dry matter

There were significant ($p \leq 0.05$) differences among treatments. Treatment 5 was significantly different from all other treatments.. There were no significant ($p \leq 0.05$) increases in nodule dry matter relative to control treatment for treatment 1, 2, and 4 (fig 3c).

Cowpea

Overall plant growth

Cowpea plants were visibly smaller than their counterparts for experiment 1. Sever discoloration of roots were observed for treatments 3 and 5. There were significant ($p \leq 0.05$) differences among treatments. Treatments 3, 4, and 5 showed significantly greater dry matter accumulation than treatments 1 and 2 (fig 4d).Treatment 3 was significant for all plant parts

Shoot dry matter

There were significant ($P \leq 0.05$) differences among treatments. Treatment 4 showed significant increases, 80%, relative to control. Uninoculated showed the least total dry matter accumulation (fig 4d). Shoot dry matter for treatment 3 was significantly different relative to control.

Root dry matter

There were significant differences among treatments. Treatment 5 was significantly different from all other treatments. Nodule dry matter appeared to be lower relative to shoot and root dry matter.

Nitrogen content

Experiment 1

Bambara groundnut

Nitrogen content in individual plant organs with nod gene-inducer treatment appear to be lower in nodules compared to shoots and roots (Fig. 5a-d). Nitrogen accumulation in the shoots, roots and nodules varied significantly ($p \leq 0.05$) with different nod gene-inducer treatments.

Shoot, roots and nodule dry matter

There were no ($p \leq 0.05$) significant detectable differences between N content among treatments for shoots. Treatments 2 and 3 showed significantly ($p \leq 0.05$) higher N content in shoots and roots relative to nodules.

Cowpea

Total nitrogen/ plant

Treatment 1, 2,3 and 4 showed significant ($p \leq 0.05$) difference in N content among treatments for total N content (Fig. 6d). N content in individual plant organs with nod gene-inducer treatment appear lower in shoots and nodules compared to roots.

Shoots, roots and nodules.

The response of nitrogen accumulation in different plant parts to nod gene-inducer treatments followed the same trend. Treatment 1 and 2 showed an increase in N content, however, treatment 5, 6 and 7 showed a significant ($p \leq 0.05$) decrease in levels compared to treatments 1 and 2 (fig 6a-c).

Experiment 2

Bambara groundnut

Total nitrogen/plant

The nitrogen content in individual plant organs with nod gene-inducer treatment appear to be lower for nodules compared to shoots or roots. At treatments 1 and 2 there appeared to be no detectable significant ($p > 0.05$) difference between nitrogen content in shoots and roots. Total N content/plant is significantly ($p \leq 0.05$) higher at treatments 3, 4 and 5 relative to treatment 1 and 2 (fig 7d).

Shoots, roots and nodules

Nitrogen accumulation in shoots, roots and nodules varied in response to nod gene-inducer treatments. Treatment 3 shows a significant ($p \leq 0.05$) increase in N accumulation for shoots, roots and nodules. However at treatments 4 and 5 a decrease in N accumulation occurred for shoots and roots while N accumulation decreased for nodule dry mass. for all plant organs treatment 5 was significantly different compared to control (fig 7a-c).

Cowpea

Total nitrogen/plant

Total nitrogen content was significantly ($p \leq 0.05$) higher among treatments. Treatment 3, 4, and 5 accumulated significantly ($p \leq 0.05$) greater nitrogen compared to the control and treatment 2. It appears that shoots and roots accumulated higher concentrations of nitrogen than the nodules (fig 8d).

Shoots, roots and nodules

Nitrogen content in shoots, roots and nodules varied significantly among treatments ($p \leq 0.05$). The response of nitrogen accumulation in the different plant organs to the nod gene-inducer treatments vary significantly ($p \leq 0.05$). For all plant organs treatment 5 was significantly higher compared to treatment 2 and the control (fig 8a-c).

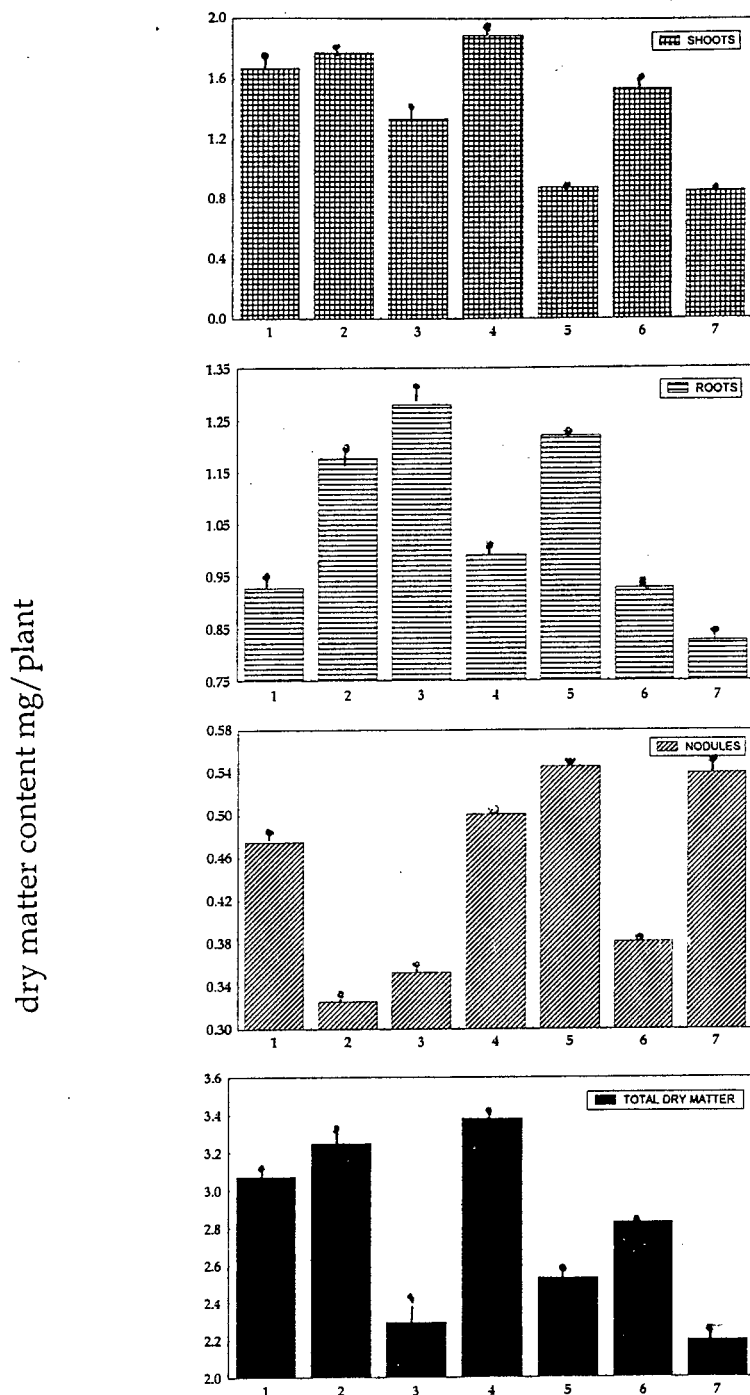


Figure 1(a-d). Dry matter of pot-grown bambara groundnut plants from experiment 1 supplied with exogenous nod gene-inducing compounds, daidzein and genistein. 1 = *Bradyrhizobium* - inoculated, 2=*Bradyrhizobium* inoculated +10 μ M daidzein, 3 = *Bradyrhizobium* - inoculated + 50 μ M daidzein, 4=*Bradyrhizobium*-inoculated + 10 μ M genistein, 5= *Bradyrhizobium*-inoculated + 50 μ M genistein, 6= *Bradyrhizobium* - inoculated + (10 μ M daidzein + 10 μ M genistein), 7= *Bradyrhizobium* - inoculated + (50 μ M daidzein + 50 μ M genistein). a= shoots, b= roots, c= nodules, d= total dry matter. N=4, \pm SD.

dry matter content mg/ plant

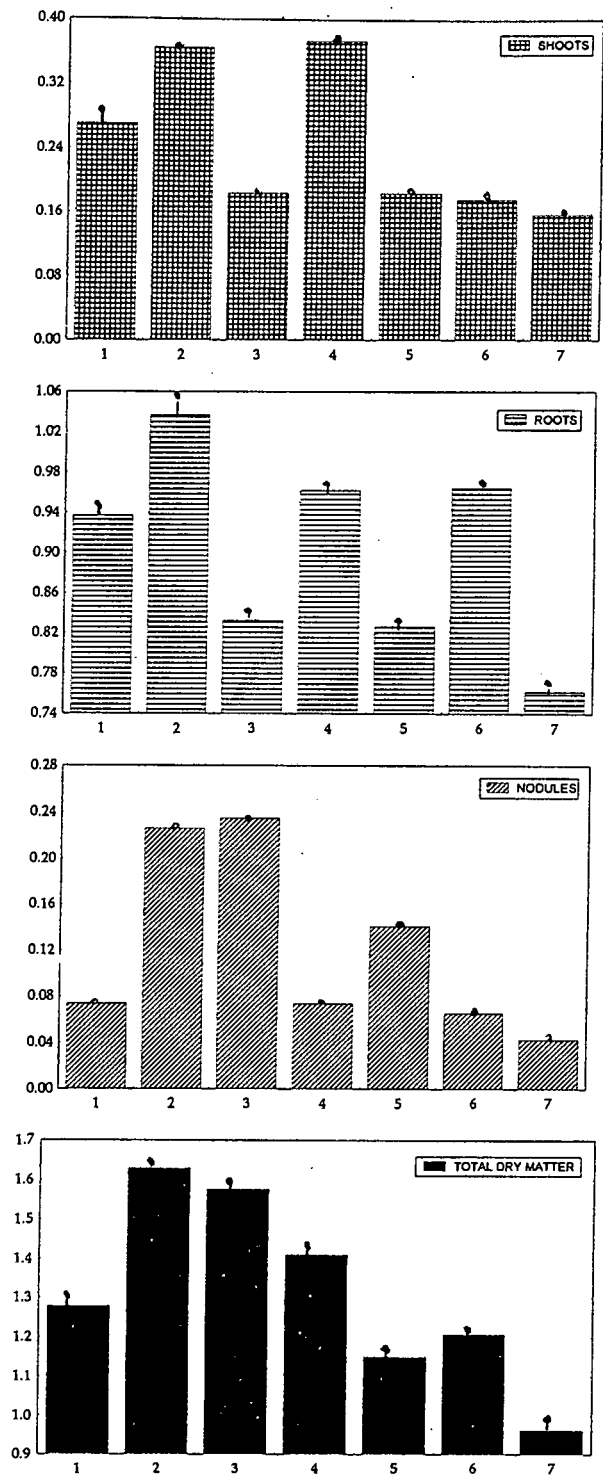


Figure 2(a-d). Dry matter of pot-grown cowpea plants from experiment 1 supplied with exogenous nod gene-inducing compounds, daidzein and genistein. 1=*Bradyrhizobium*-inoculated, 2=*Bradyrhizobium* -inoculated +10 μ M daidzein, 3= *Bradyrhizobium*-inoculated + 50 μ M daidzein, 4= *Bradyrhizobium*-inoculated + 10 μ M genistein, 5= *Bradyrhizobium*-inoculated + 50 μ M genistein, 6= *Bradyrhizobium*-inoculated + (10 μ M daidzein + 10 μ M genistein), 7= *Bradyrhizobium*-inoculated + (50 μ M daidzein + 50 μ M genistein). a= shoots, b= roots, c= nodules, d= total dry matter. N=4, \pm SD.

dry matter content mg/plant

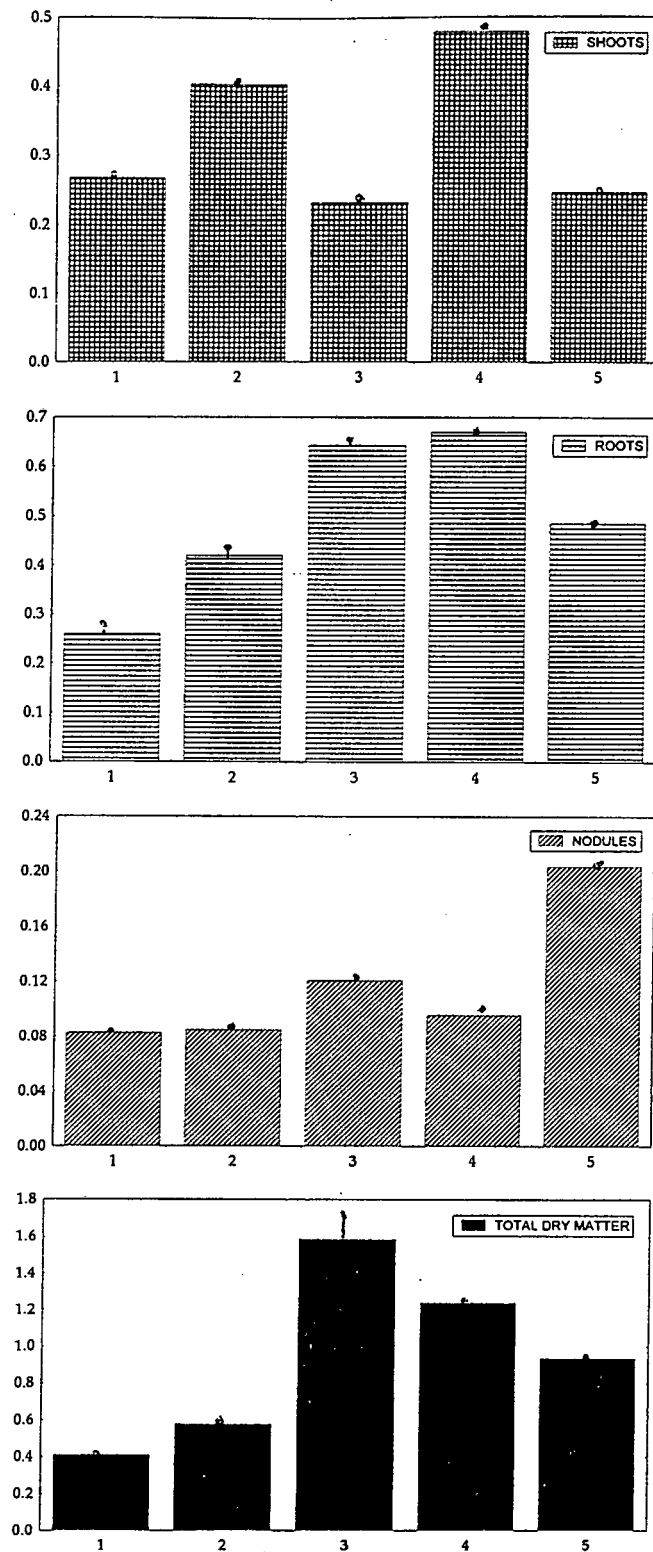


Figure 3 (a-d). Dry matter of Leonard-jar-grown bambara groundnut plants from experiment 2 supplied with exogenous nod gene-inducing compounds, daidzein and genistein. 1=uninoculated, 2=*Bradyrhizobium*-inoculated, 3=*Bradyrhizobium*-inoculated + 50 μ M daidzein, 4=*Bradyrhizobium*-inoculated + 50 μ M genistein, 5=*Bradyrhizobium*-inoculated + (50 μ M daidzein + 50 μ M genistein). a= shoots, b= roots, c= nodules, d= total dry matter. N=4, \pm SD.

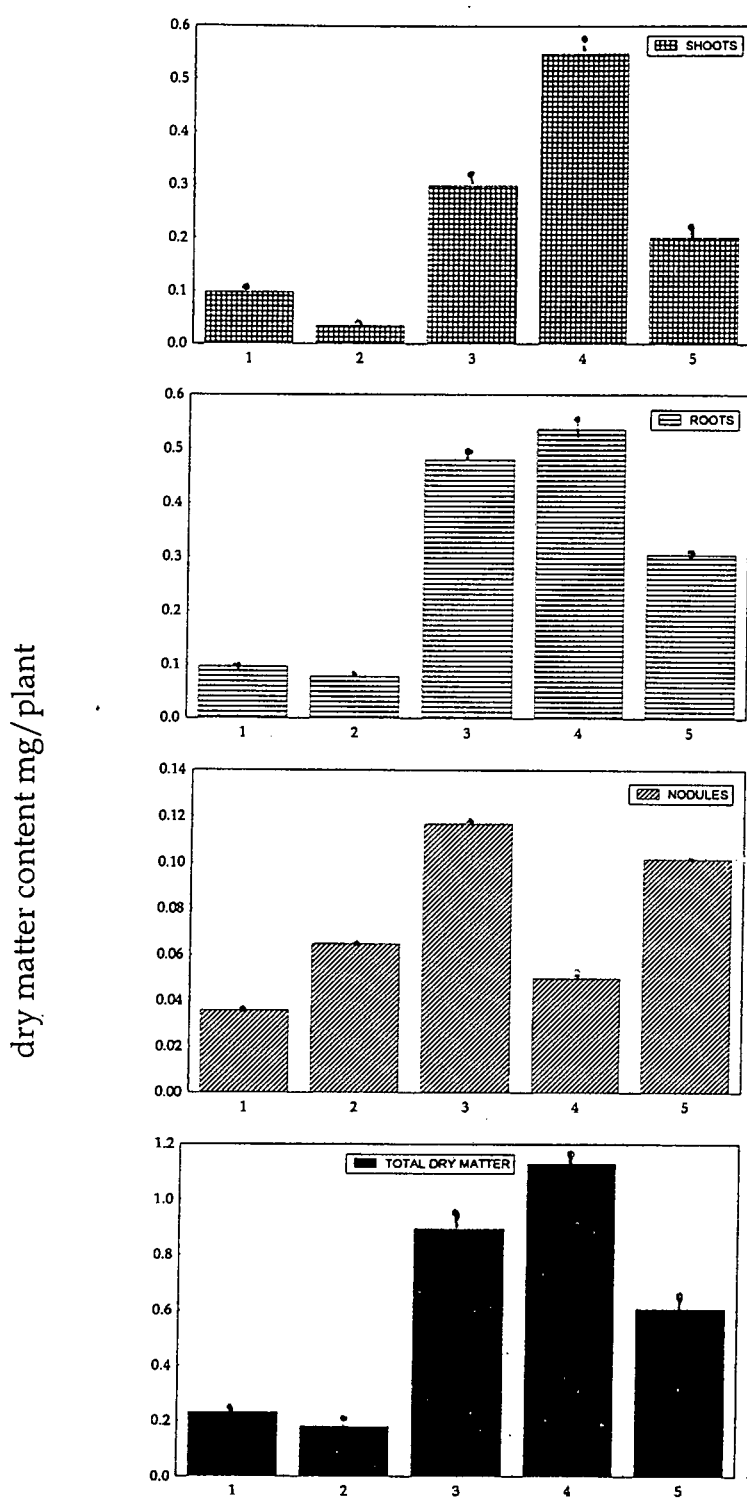


Figure 4(a-d). Dry matter of Leonard jar-grown cowpea plants from experiment 2 supplied with exogenous nod gene-inducing compounds, daidzein and genistein. 1=uninoculated, 2= *Bradyrhizobium*-inoculated, 3= *Bradyrhizobium*-inoculated + 50 μ M daidzein, 4= *Bradyrhizobium*-inoculated + 50 μ M genistein, 5= *Bradyrhizobium*-inoculated + (50 μ M daidzein + 50 μ M genistein). a= shoots, b= roots, c= nodules, d= total dry matter. N=4, \pm SD.

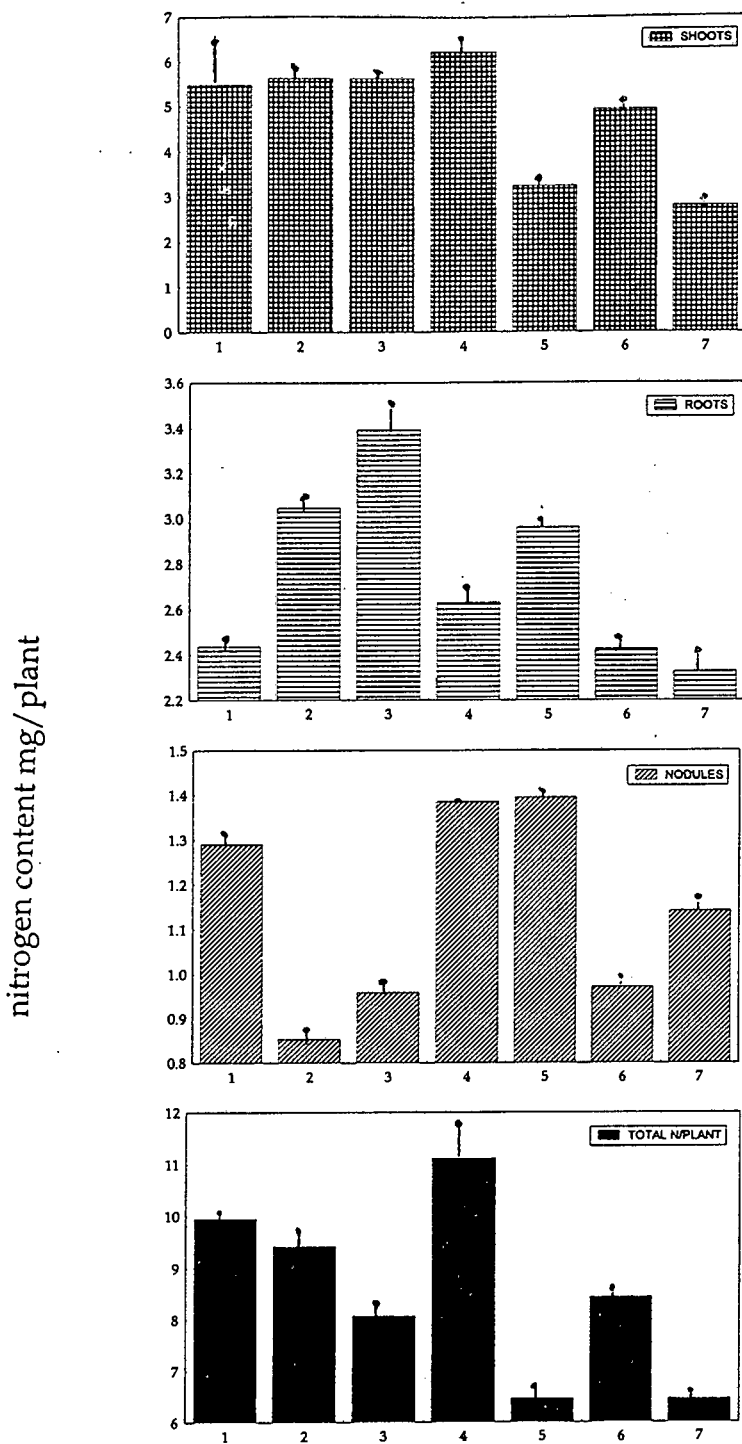


Figure 5 (a-d). Nitrogen content of pot-grown bambara groundnut plants from experiment 1 supplied with exogenous nod gene-inducing compounds, daidzein and genistein. 1=*Bradyrhizobium*-inoculated, 2=*Bradyrhizobium* -inoculated +10 μM daidzein, 3= *Bradyrhizobium*-inoculated + 50 μM daidzein, 4= *Bradyrhizobium*-inoculated + 10 μM genistein, 5= *Bradyrhizobium*-inoculated + 50 μM genistein, 6= *Bradyrhizobium*-inoculated + (10 μM daidzein + 10 μM genistein), 7= *Bradyrhizobium*-inoculated + (50 μM daidzein + 50 μM genistein). a= shoots, b= roots, c= nodules, d= total dry matter. N=4, \pm SD.

nitrogen content mg/plant

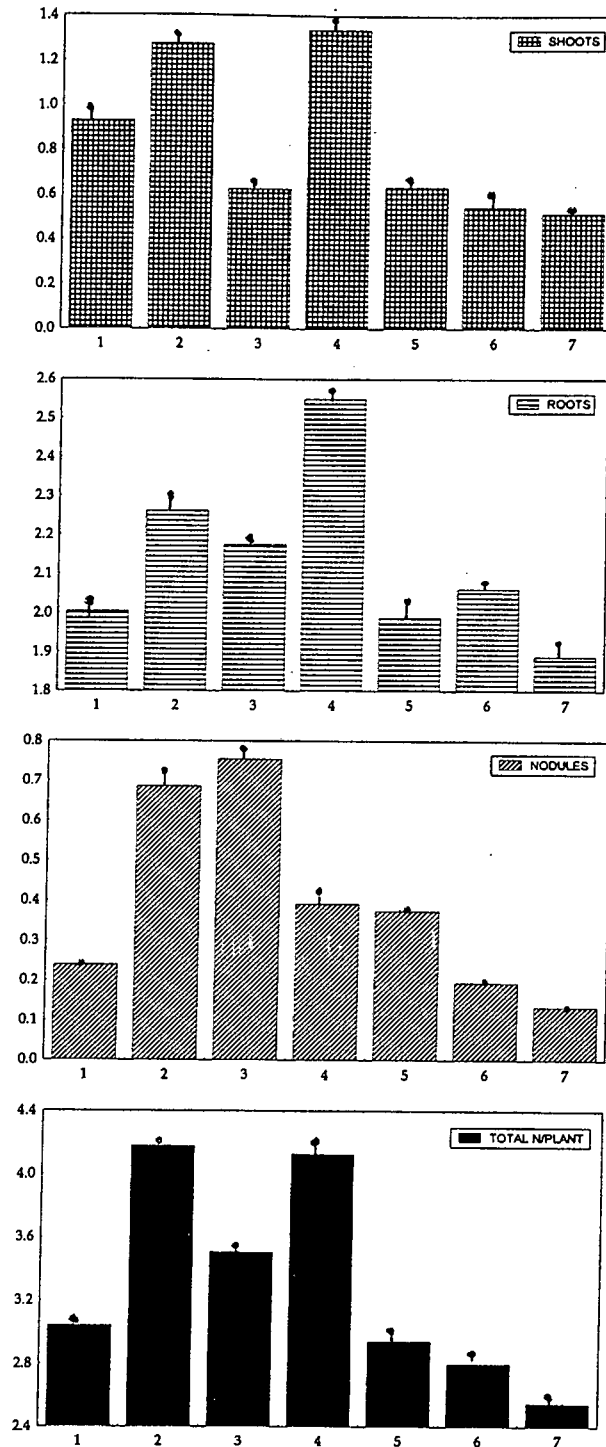


Figure 6(a-d). Nitrogen content of pot-grown cowpea plants from experiment 1 supplied with exogenous nod gene-inducing compounds, daidzein and genistein. 1=*Bradyrhizobium*-inoculated, 2=*Bradyrhizobium*-inoculated + 10 μ M daidzein, 3= *Bradyrhizobium*-inoculated + 50 μ M daidzein, 4= *Bradyrhizobium*-inoculated + 10 μ M genistein, 5= *Bradyrhizobium*-inoculated + 50 μ M genistein, 6= *Bradyrhizobium*-inoculated + (10 μ M daidzein + 10 μ M genistein), 7= *Bradyrhizobium*-inoculated + (50 μ M daidzein + 50 μ M genistein). a= shoots, b= roots, c= nodules, d= total dry matter. N=4, \pm SD.

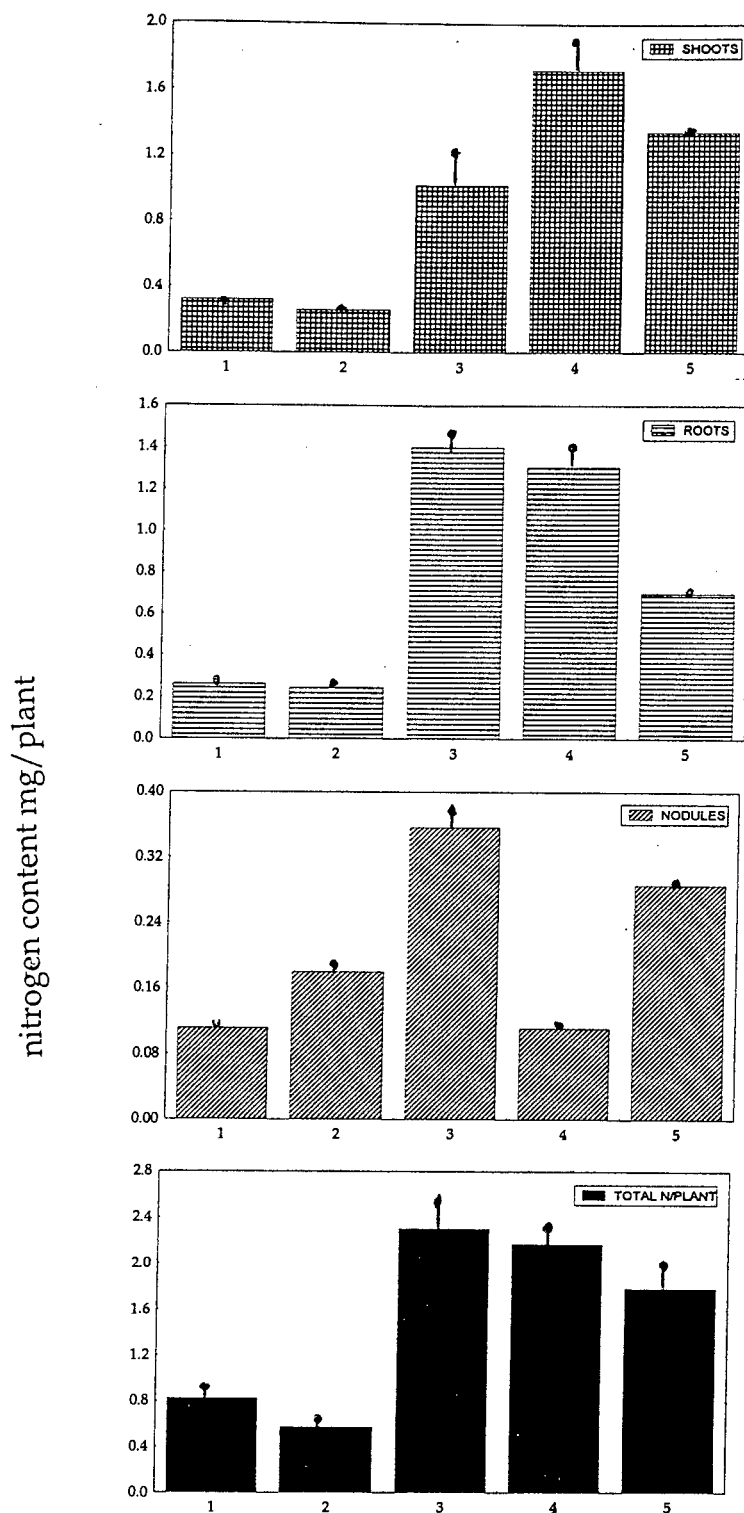


Figure 7(a-d). Nitrogen content of Leonard jar-grown bambara groundnut plants from experiment 2 supplied with exogenous nod gene-inducing compounds, daidzein and genistein. 1=uninoculated, 2=*Bradyrhizobium* -inoculated, 3= *Bradyrhizobium*-inoculated + 50 μ M daidzein, 4= *Bradyrhizobium*-inoculated + 50 μ M genistein, 5= *Bradyrhizobium*-inoculated + (50 μ M daidzein + 50 μ M genistein). a= shoots, b= roots, c= nodules, d= total dry matter. N=4, \pm SD.

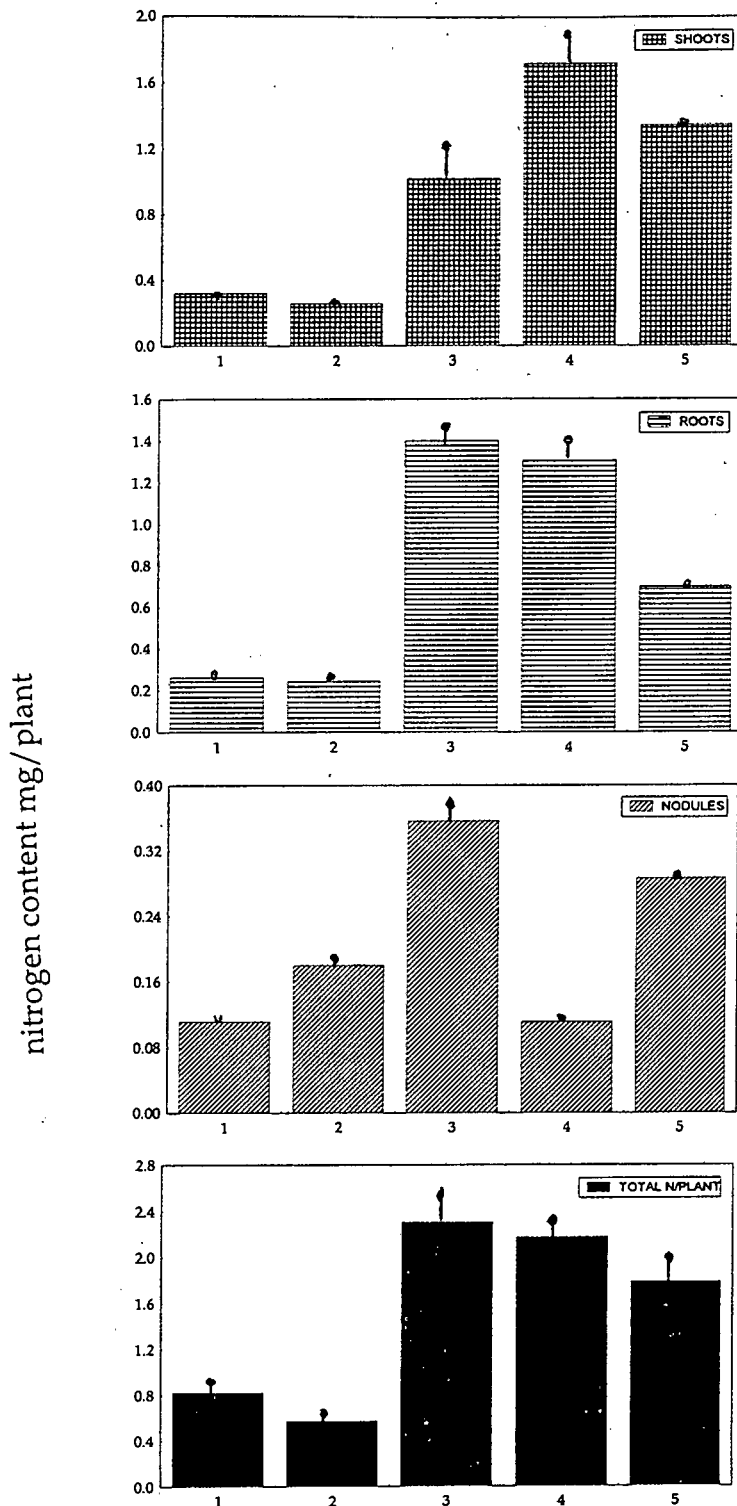


Figure 7(a-d). Nitrogen content of Leonard jar-grown bambara groundnut plants from experiment 2 supplied with exogenous nod gene-inducing compounds, daidzein and genistein. 1=uninoculated, 2=*Bradyrhizobium*-inoculated, 3= *Bradyrhizobium*-inoculated + 50 μ M daidzein, 4= *Bradyrhizobium*-inoculated + 50 μ M genistein, 5= *Bradyrhizobium*-inoculated + (50 μ M daidzein + 50 μ M genistein). a= shoots, b= roots, c= nodules, d= total dry matter. N=4, \pm SD.

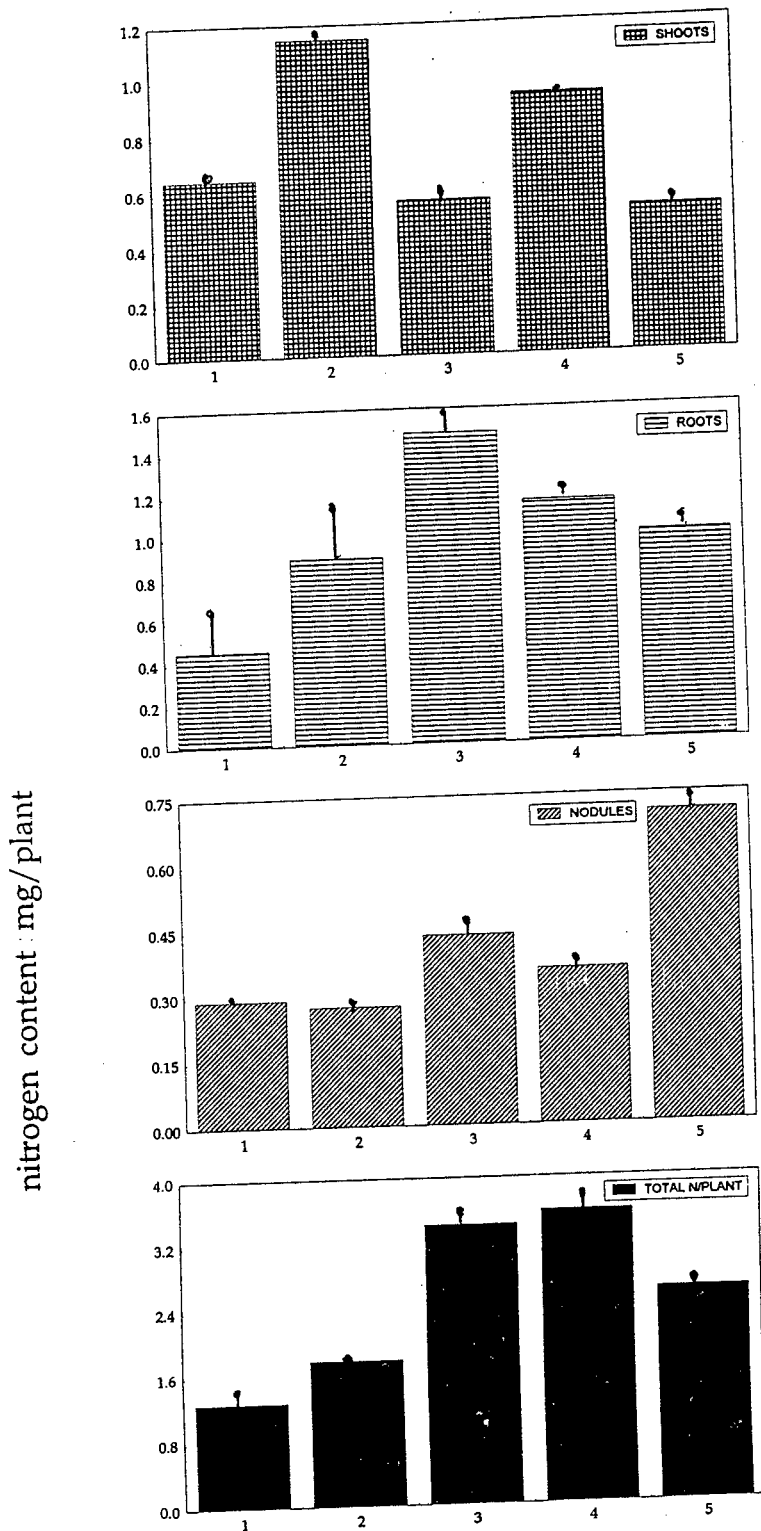


Figure 8(a-d). Nitrogen content of Leonard jar-grown cowpea plants from experiment 2 supplied with exogenous nod gene-inducing compounds, daidzein and genistein. 1=uninoculated, 2=*Bradyrhizobium*-inoculated, 3=*Bradyrhizobium*-inoculated + 50 μ M daidzein, 4= *Bradyrhizobium*-inoculated + 50 μ M genistein, 5= *Bradyrhizobium*-inoculated + (50 μ M daidzein + 50 μ M genistein). a= shoots, b= roots, c= nodules, d= total dry matter. N=4, \pm SD.

DISCUSSION

Scientific research has shown that daidzein and genistein are potent inducers of nodulation genes in *B. japonicum*. However daidzein has shown to have less nod gene-inducing ability than genistein (Sutherland *et al.*, 1990). Several studies have shown that supplementary treatment with nod gene-inducers increase root nodulation (Hungria and Phillips, 1993; Dakora and Nyamekye, 1997).

For experiment 1 both species showed a maximum response to nodule dry matter at 10 μM daidzein. This response was statistically significant ($p \leq 0.05$) Applied 10 μM daidzein resulted in a 17% increase in total dry matter. This revealed that total biomass and nodulation was enhanced by applied nod gene-inducer. Treatments 5 to 7 were outperformed by the control for total dry matter for both species, The data suggests that plant biomass was at least enhanced by 10 μM daidzein, 50 μM daidzein and 10 genistein. Bambara groundnut shoot dry matter was reduced at 50 μM daidzein, 50 μM genistein and combined 50 μM of daidzein and genistein. Similarly Dakora and Nyamekye (1997) reported that bambara groundnut shoot growth is significantly reduced when nod gene-inducers were applied. However, these results show that shoot growth is significantly reduced at high nod gene concentrations. Thus high concentrations of nod gene-inducers can affect nodulation and nitrogen fixation. Graham (1991) suggests that soybean seeds may possess a feedback mechanism to regulate the exudation of its very large stores of isoflavonoid signal molecules. The release of small amounts is perhaps indicative of the effectiveness with respect to nodulation. Some researchers have shown that nitrogen fixation and nodulation can be increased significantly when treated with nod gene-inducers. Dr D. Smith have

suggested that high concentrations of these compounds have a negative effect on the bacteria as well as plant organs (personal communication). The fact that the plant roots does not secrete all its reserve flavonoids is an indication that: (i) the bacteria are toxic to these molecules or (ii) the plant secretes only the amount it requires for nodulation to occur. Since concentrations released by roots is so low for experiment 1, both total dry matter, nodule dry mass and N content significantly differed between 10 μM daidzein and 50 μM daidzein. Total N content and nodule accumulation decreased from treatment 4 to 7 compared to control. Therefore nod gene-inducer application is not generally enhanced for various concentrations and combinations. For experiment 1, applied 10 μM genistein significantly increased total dry matter and nitrogen accumulation compared to control. Zhang and Smith, (1995) reported that total plant biomass as well as nodule number was reduced at temperatures above 25°C at 30 μM genistein. Therefore concentrations of nod gene-inducers are a significant factor as well as environmental conditions.

For experiment 2 both bambara groundnut and cowpea showed significant increases in total dry mass and nitrogen (N) accumulation for preincubation with treatment 5 (combined 50 μM daidzein and 50 μM genistein). Treatment 5 was significantly different from the control for both species. Also at 50 μM daidzein nitrogen accumulation for nodule and nodule dry matter were significantly greater compared to control. 50 μM genistein showed a significant increase in shoot dry matter at 21% relative to control. However at 50 μM genistein, nodule mass consistently decrease for both species. Hungria and Stacey, (1997) reported that pre-treatment of *Rhizobium*

with genistein increased nodulation and plant growth for field grown peanut (*Arachis hypogea*). Bean or soybean seeds inoculated and treated with 40 μM genistein produced plants with significantly higher nodule number. These results suggests that routine increases in nodulation of some legume species is possible when exogenous nod gene-inducers are applied. However, Zhang and Smith (1995) reported that for soybean a decrease in nodule number was observed after concentrations at 30 μM genistein or higher. For both species nodular N content and nodular dry matter did not differ significantly between uninoculated and inoculated treatments for experiment 2. Total dry matter were not significantly different between uninoculated and inoculated treatments.

Genistein appears to have no significant effect on field grown plants. Temperature appears to play a role.. It could also be degraded by soil micro-organisms. Several reports give evidence to show that (*Brady*)*Rhizobium* degrades flavonoids as well as isoflavonoids molecules (Graham, 1991, Zhang and Smith, 1995). It seems, generally , that daidzein is the better nod gene inducer and doubles as a phytoalexin as well (Dakora and Phillips, 1996). It is also located within the root where root hair emergence is initiated (Graham, 1995).

Genistein is easily degraded by rhizobia via hydrolyses of the C-ring (Rao and Cooper, 1994) and probably catabolised by rhizobia (Dakora and Nyamekye). In experiment 2, both species show consistently high nodule mass and nitrogen accumulation when preincubated with both daidzein and genistein. Nitrogen content and nodule dry mass was increased when preincubated with daidzein alone whereas preincubation with genistein was ineffective.

Rao and Cooper (1995) reported that daidzein concentrations was enhanced after incubation with *B. japonicum*. In contrast, the amount of genistein declined after rhizobial incubation compared to inoculant free control and inoculant (treatment 2).

Their results concur with this data in that nodulating rhizobia can metabolise significant quantities of nod gene-inducing isoflavones daidzein and genistein via similar modes of biotransformation.

Common bean root exudates are also known to contain higher levels of coumestrol and daidzein in response to *Rhizobium* inoculant (Dakora *et al.*, 1993).

Data from this study indicate that substantial changes in the inducing activity of isoflavonoids occur during preincubation with rhizobia. The accumulation of daidzein and/or genistein and their metabolic derivatives in the cells raises the possibility of competitive binding to nodD proteins as a mechanism for regulatory nod gene expression.

Our data suggests a specific effect of degradation products on nod gene induction rather than a general toxicity towards growth of organism.

Rao and Cooper (1995) failed to detect toxic effects from a variety of phenolic compounds.

It is possible that metabolic derivatives of daidzein and genistein such as the open C-ring isoliquiritigen. Isoliquiritigen offers increased molecular flexibility to match the optimal conformation of nodD receptor protein required for nod gene regulation. Rao and Cooper have speculated that potent nod gene inducers could have a dual function in symbiotic communication, as is suggested by treatment 5 (combined nod gene-inducers). Further studies at the subcellular level would be required to verify this suggestion

CONCLUSION

In answering the initial questions for experiment 1: bambara groundnut nodule mass was significantly increased when either genistein concentrations was applied. Although total dry mass decreases at this treatment. Applied daidzein concentrations resulted in a significant decrease in nodule dry mass. For cowpea it is just the opposite. Applied concentrations of daidzein significantly increases nodule dry mass. whereas genistein concentrations decrease nodule dry mass.

For experiment 2, although bambara groundnut nodule mass appear slightly higher at 50 μ M daidzein, it is not significantly different from 50 μ M genistein. For cowpea preincubated 50 μ M daidzein results in a significantly higher nodule dry mass.

Does adding nod gene-inducers promote nodulation? Nodulation is promoted, however, concentration, environmental conditions, and method of inoculation can affect the inductive effect of nod gene-inducers.

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