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Growth media effects on shoot physiology, nodule numbers and symbiotic nitrogen fixation in soybean

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Abstract

Several research groups (both in South Africa and other countries) are currently involved in research aimed at improving symbiotic nitrogen fixation (SNF) and root nodule sustainability in soybean [*Glycine max* (L.) Merr.]. In many of these experiments potted plants are used, and in this paper the importance of careful selection of growth media is demonstrated. *Bradyrhizobium japonicum*-inoculated soybean seedlings were cultivated in pots containing N-free growth media (sand, fine vermiculite or coarse vermiculite) or a growth medium containing low concentrations of water-soluble nitrogen predominantly in the form of ammonium (mixture of potting soil, sand and vermiculite). The effects of growth media on shoot physiology were assessed by measurement of plastochron index, chlorophyll content and CO_2 assimilation rates. Nodule numbers, nitrogenase activity and nodule ureide content were also determined. Although similar source–sink relationships were maintained in plants cultured in the various growth media, large effects on nodule numbers and SNF were observed. Shoot phenotype and physiology did not provide any insight into these belowground effects. The presence of mineral N, or sand as culture medium, led to the formation of more abundant nodules but with low SNF activity. Vermiculite, irrespective of particle size, resulted in plants with root systems housing nodules with high SNF activity. It is concluded that choice of growth media for cultivating soybean plants under controlled growth conditions is an important consideration, especially in multi-institution collaborations where comparability between experiments is a pre-requisite.

Keywords: Nitrogenase; Nodulation; Root nodules; Soybean [*Glycine max* (L.) Merr.]; Symbiotic nitrogen fixation; Ureides

1. Introduction

Symbiotic nitrogen fixation (SNF) by annual legume crops such as soybean plays a key role in the maintenance of global food production (Bordeleau and Prévost, 1994; Herridge and Rose, 2000). It has been realised for some time that enhancement of SNF in agriculture would become increasingly important as N-fertiliser derived from fossil fuels becomes more expensive (LaRue, 1978).

The symbiotic association between the roots of soybean and *Bradyrhizobium japonicum* bacteria leads to formation of specific organs, called root nodules, where SNF takes place. The main products of SNF in soybean root nodules, namely ureides (allantoin and allantoic acid), are exported to the rest of the plant where it is incorporated into amino acids and proteins. The establishment and maintenance of an effective symbiosis depends on several factors of which a favorable environment, that will allow maximum N_2 fixation, is extremely important (Bordeleau and Prévost, 1994). Several environmental factors such as soil pH, soil fertility, drought stress and temperature extremes impose limitations on the symbiotic association between the host plant and microsymbiont (Vessey and Waterer, 1992; Bordeleau and Prévost, 1994; Serraj et al., 1999; Van Heerden et al., 2003).

Even under optimal growth conditions the functional lifespan of root nodules is relatively short where after the symbiosis is terminated through a process known as nodule senescence. In the case of soybean nodules, senescence begins at the center of the nodule and progressively spreads to the outside. A major problem in agriculture is that nodule senescence can be induced prematurely by various factors such as temperature extremes and drought stress (Gogorcena et al., 1995; Gonzalez et al., 1998).

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Various observations suggest that shoot and root derived signals are major determinants of the nodule senescence process, but the factors that orchestrate this process, and the identity of the signals involved, have received relatively little attention (Puppo et al., 2005).

Because of the problems associated with premature nodule senescence and the agricultural importance of soybean, it is not surprising that several research groups (both in South Africa and other countries) are currently involved in research aimed at improving soybean production, SNF and nodule sustainability. In many of these experiments potted soybean plants are used, and in this paper it is demonstrated how different growth media markedly affects nodule numbers and SNF without any apparent effects on shoot development and physiology. A very sensitive indicator of shoot development, namely the plastochron index (Erickson and Michelini, 1957) was used to quantify any effects on shoot growth. The time interval between the initiation of consecutive leaves on a plant is termed the "plastochron". The plastochron index (PI) is considered a very sensitive indicator of plant development and has been used extensively for various physiological investigations, especially in legumes grown under controlled environmental conditions (Snyder and Bunce, 1983; Yourstone and Wallace, 1990; Jamadagni et al., 1995; Ade-Ademilua and Botha, 2004). Among its applications, the PI enables precise quantification of shoot growth rates (Snyder and Bunce, 1983). The results reported in this paper emphasise the need for very careful growth media selection and standardisation, especially in the case of multi-institution collaborations where uniform plant culture among the various participating laboratories is an absolute prerequisite for studies on nodulation and symbiotic nitrogen fixation.

2. Materials and methods

2.1. Plant growth

Seeds of the South African soybean genotype PAN809 were sown in 2 dm^3 plastic pots containing the following growth media: (a) sterile river sand; (b) fine vermiculite (FV;±2 mm diameter); (c) coarse vermiculite (CV;±5–7 mm diameter) and (d) potting soil without added fertiliser (Culterra, PO Box 982, Muldersdrif 1747, South Africa) mixed with river sand and vermiculite (ratio of 4:2:1 respectively). The total water soluble N-content of this potting soil mixture (PSM) was 4.9 mg/l whereas the other growth media can be regarded as N-free. In all cases the seeds were inoculated with *Bradyrhizobium japonicum* (bacterial strain WB 74) at the time of sowing to ensure root nodule development and SNF. Seedlings were grown in a Conviron PGV 36 growth chamber (Controlled Environments Ltd., Winnipeg, MB, Canada, R3H 0R9) under rigorously controlled growth conditions: 15 h/9 h and 26 °C/20 °C light/dark cycle with a light intensity of 1000 μmol m^{-2} s^{-1} at the level of the plant canopy. Illumination was provided by a combination of fluorescent (Sylvania Cool White VHO, 215 W) and incandescent (Sylvania, 100 W) lamps. The incandescent lamps were included as an enriching source of red light to minimise any growth abnormalities known to occur when plants are grown under artificial illumination. The fluorescence lamps were replaced on a regular basis to ensure maximal and comparable light intensities during all experiments. Potted plants were rotated daily to compensate for any variations in light intensity, especially along the sides of the growth chamber. Seedlings were watered daily and supplied with N-free (to prevent inhibition of SNF) full-strength Hoagland nutrient solution (Hoagland and Arnon, 1950) three times a week. Two seeds were sown per pot but seedlings were thinned out to one plant per pot after emergence to minimise effects that the pots could have on plant development.

2.2. Quantification of vegetative development

The vegetative development of the plants cultivated in the four different growth media (sand, FV, CV and PSM) was quantified by repeated measurements of the plastochron index (Erickson and Michelini, 1957). All trifoliate leaves with central leaflets exceeding a reference length of 25 mm (Snyder and Bunce, 1983) were counted. The length of the youngest central leaflet longer than or equal to 25 mm, as well as the length of the central leaflet (shorter than 25 mm) on the next trifoliate leaf was measured. The PI of each plant was calculated using the following formula:

$$\mathrm{PI} = n + (\log L_n - \log L_{\mathrm{ref}})/(\log L_n - \log L_{n+1})$$

where n = number of trifoliate leaves with central leaflets longer than the reference length of 25 mm (L_{ref}), L_n = length of the central leaflet on trifoliate leaf L_n (which by definition is longer than or equal to L_{ref}) and L_{n+1} = length of the central leaflet on trifoliate leaf L_{n+1} (which by definition is shorter than L_{ref}). Shoot growth rates (SGR = increase in plastochron index units per day) during the experimental period (between 12 and 32 days after sowing) were determined by calculating the slopes of linear regressions constructed by plotting plastochron index values for each growth media treatment against time. In all cases the increase in plastochron index values over time was highly linear ($r^2 > 0.96$) validating the use of this method for determination of SGRs.

2.3. Measurement of chlorophyll content

In a separate experiment, the chlorophyll content of leaf discs harvested from soybean leaves of different stages of development (young to senescent) were measured with a hand-held chlorophyll Content Meter (CCM-200, Opti-Sciences, Inc., 164 Westford Road #4, Tyngsboro, MA 01879, USA), which recorded a chlorophyll content index (CCI) for each leaf disc. The total extractable chlorophyll content of each of these leaf discs was determined according to the method of Wintermans and De Mots (1965). The actual chlorophyll content of each leaf disc was plotted against its corresponding CCI value. This calibration curve revealed a highly linear relationship ($r^2 > 0.97$) between actual chlorophyll content and a wide range of recorded CCI values, thus justifying the use of the measured index values. In the growth media experiments, the CCI values of the first trifoliate leaf of plants were measured at regular intervals during leaf development to assess any effects on chlorophyll content.

2.4. Measurement of CO_2 assimilation

CO_2 assimilation measurements were conducted on the same leaves with a portable photosynthesis system (CIRAS-2, PP-Systems, Hertz, UK) 32 days after sowing (average plastochron index of 8.4). All measurements were conducted at a leaf temperature of 26 °C using a photosynthetic leaf chamber (PLC) featuring light, temperature and humidity control. Humidity in the PLC was maintained close to ambient conditions. Irradiance during measurements was controlled at 1200 μmol photons m^{-2} s^{-1}. Whilst in the PLC, each leaf was exposed to a series of increasing atmospheric CO_2 concentrations (C_a). At each C_a level, the ratio of CO_2 assimilation rate (A) to intercellular CO_2 concentration (C_i) was automatically recorded. By increasing C_a at 5-min intervals from 0 to 2000 μmol/mol, A:C_i response curves were generated for each leaf. The initial slope of the demand function ($\delta A/\delta C_i$) was computed by linear regression analysis. All other calculations were done according to Farquhar and Sharkey (1982) and Chaves (1991).

2.5. Nitrogenase activity

Directly after the CO_2 assimilation measurements, four plants of each treatment were removed from the pots and the root systems carefully rinsed to remove most of the adhering growth media. Whole root systems with attached nodules were incubated for 10 min in 250 ml flasks in the presence of 1% (v/v) acetylene for the measurement of nitrogenase activity with the acetylene reduction assay (Turner and Gibson, 1980). Although nitrogenase may be inhibited to some extent by acetylene (Minchin et al., 1983), comparative measurements over a short assay-time are acceptable and provide reliable data. In addition, we established that the presence of acetylene inhibit nitrogenase activity only after incubation times exceeding 20 min under the assay conditions employed in these experiments.

2.6. Nodule ureide content

Immediately after the acetylene reduction assay, all the nodules were removed from the roots, counted, weighed and dried at 60 °C for measurement of ureide (allantoin and allantoic acid) content. Ureides were extracted from nodules with 1 ml 0.2 M NaOH followed by boiling for 20 min. After centrifugation (10 min at 10,000 ×g) the ureide content of each clarified supernatant was determined colorimetrically (525 nm) in the presence of HCl/Phenylhydrazine and HCl/KFeCn according to the method described by Young and Conway (1942). Ureide content of samples was estimated from a standard curve ranging between 0 and 8 μg allantoin.

2.7. Statistical analysis

Statistical analysis was conducted with the software package Statistica for Windows version 6 (StatSoft, Inc. 2300 East 14th Street, Tulsa OK 74104, USA). Normal distribution of data was determined using the Shapiro–Wilk W test. In data sets with parametric distribution, significant differences between treatment means were determined using Student's t-test. In data sets with non-parametric distribution, significant differences between treatment means were determined with the Mann–Whitney U-test.

3. Results and discussion

Cultivation of soybean seedlings in the different growth media did not induce any visible differences in shoot phenotype (Fig. 1). The visual observations were confirmed by repeated measurements of plastochron index over time (Fig. 2). These measurements revealed that the plants of all four treatments had plastochron index values not significantly different ($p>0.05$) from each other at the end of the experimental period (32 days after sowing). The plants of the different treatments also had very similar SGRs of between 0.27 and 0.29 plastochron index units per day. Taken together, our results show that cultivation of plants in the four different growth media had no effects on shoot growth and development.

Non-destructive measurement of chlorophyll content during the development of individual leaves was used as an indicator of shoot N-status. The use of chlorophyll meters to predict N-content of leaves has been reported many times and generally a good correlation between chlorophyll content and leaf N-content is obtained (e.g. Reeves et al., 1993; Bullock and Anderson, 1998). The change in chlorophyll content over time in first trifoliate leaves of plants of the various treatments is shown in Fig. 3. The progressive increase in chlorophyll content during leaf expansion until maximum values, which coincided with full leaf development approximately four weeks after sowing, is clearly demonstrated. The developmental pattern in leaves were very similar for all growth media treatments and in addition, actual chlorophyll content values between treatments did not differ significantly from each other. These results show indirectly that the N-status of the plants of all four treatments was very similar and that the presence of low levels of mineral nitrogen in the PSM treatment at the time of sowing did not convey any advantage in terms of shoot growth rates (Fig. 2) or chlorophyll content (Fig. 3). Our findings are different to previous observations where it was demonstrated that soybean plants cultivated in the presence of ammonium had considerably higher whole-plant dry weights and total N accumulation than plants fertilised with N-free nutrient solution (Gulden and Vessey, 1998). The most probable explanation for this difference is that the low levels of water-soluble nitrogen in the PSM were gradually leached out during regular watering. It must also be remembered that these plants, similar to the other three treatments, were fertilised with N-free nutrient solution.

The similarities in shoot physiology of the plants cultivated in the four different growth media were further confirmed through measurement of CO_2 assimilation rates. The CO_2 response curves recorded in these plants (Fig. 4) revealed very similar kinetics in terms of carboxylation efficiency (CE) and CO_2 saturated rates of photosynthesis (J_{max}). The initial slope of the CO_2 response curve is a reliable indicator of *in vivo* ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity, while J_{max} reflects the ribulose-1,5-bisphosphate (RuBP) regeneration capacity of the leaf (Farquhar and Sharkey,

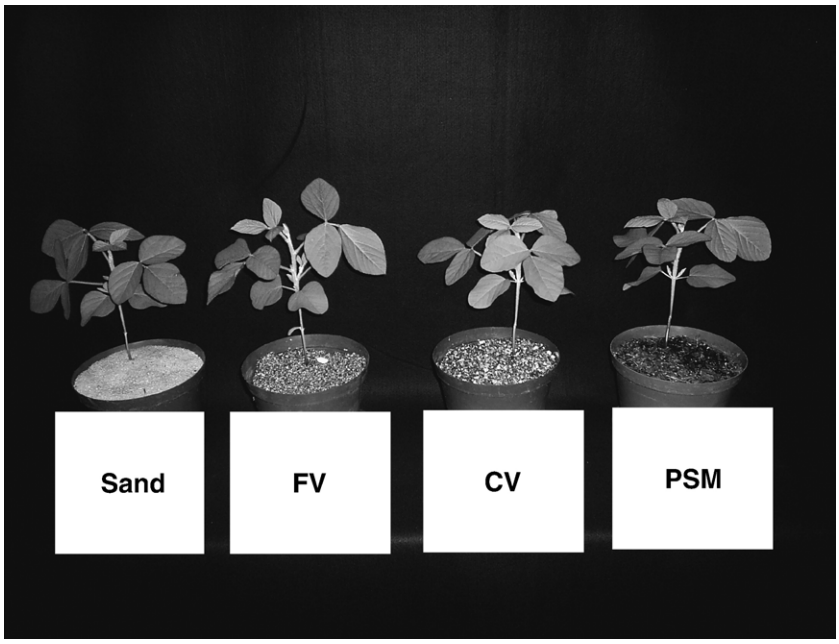

Fig. 1. Shoot phenotypes of soybean seedlings cultivated in sand, fine vermiculite (FV), coarse vermiculite (CV) and potting soil mixture (PSM). Seeds were inoculated with *Bradyrhizobium japonicum* at the time of sowing to ensure root nodule formation. Seedlings were provided with N-free nutrient solution at regular time-intervals during the growth period.

1982). Overall, these measurements indicate very similar Rubisco activities, RuBP regeneration by the stromal bisphosphatases, and reducing-equivalent supply through photosynthetic electron transport in all treatments. Because of the similarities in shoot growth, chlorophyll content and CO_2 assimilation capacity on a leaf area basis, we conclude that photoassimilate production in plants of all four treatments was very similar.

In contrast to the similarities in shoot physiology, the four different growth media had pronounced effects on nodule numbers (Fig. 5A), nitrogenase activity (Fig. 5B) and nodule ureide content (Fig. 5C). Root systems of plants cultivated in sand and PSM had more than double the amount of nodules compared to plants cultivated in FV and CV (Fig. 5A). On the other hand, nodule metabolic activity, as assessed by measurement of nitrogenase activity on a nodule weight basis, was much higher in the FV and CV treatments (Fig. 5B). These higher nitrogenase activities were further confirmed by ureide contents nearly double that of plants cultivated in the sand and PSM treatments (Fig. 5C). The clear inverse relationship between nodule numbers and nitrogenase activity is illustrated in Fig. 6. The nitrogenase activity of nodules on roots systems containing abundant nodules was clearly much lower than those of nodules on root systems with less-abundant nodules. Taken together,

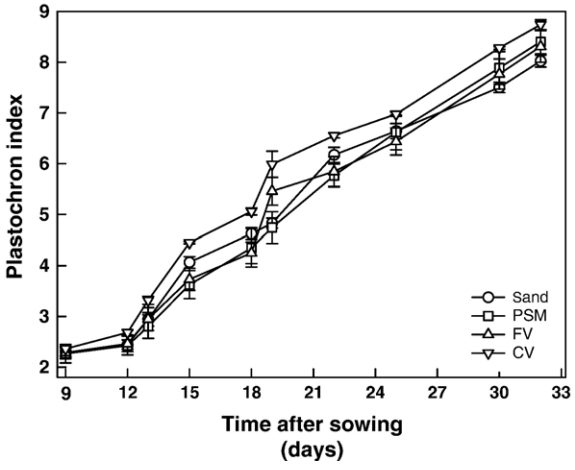

Fig. 2. Shoot development, as quantified with the plastochron index, in soybean seedlings cultivated in the four different growth media. Each data point represents the mean of four replicates ± standard error.

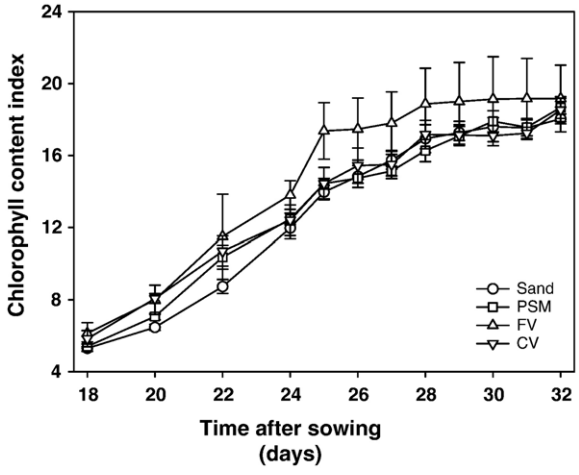

Fig. 3. Chlorophyll contents of the first trifoliate leaves of soybean seedlings cultivated in the four different growth media. The chlorophyll content of these leaves was routinely determined from early leaf expansion through to full leaf development. Each data point represents the mean of four replicates ± standard error.

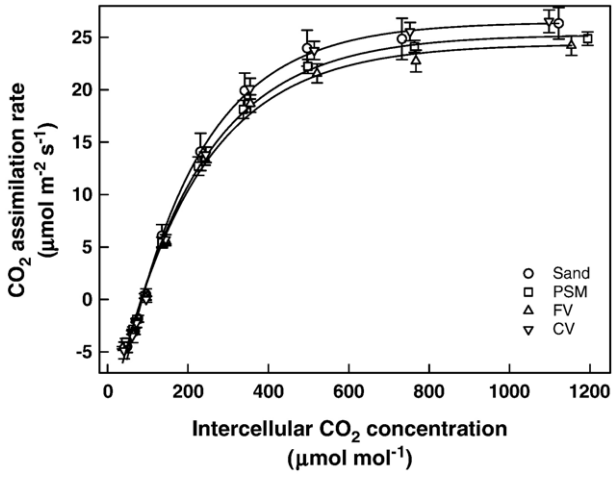

Fig. 4. Photosynthetic capacities of the first trifoliate leaves of soybean seedlings cultivated in the four different growth media. CO_2 response curves were generated in these leaves 32 days after sowing. Each data point represents the mean of four replicates ± standard error.

these results indicate that sand and PSM resulted in low SNF activity on a nodule weight basis, but that this limitation was balanced-out by large increases in nodule numbers. This compensatory response ensured maintenance of similar source–sink relationships in these plants as indicated by the similar shoot growth rates, chlorophyll contents and photosynthetic capacities compared to plants cultivated in CV or FV. The differences in physical properties (e.g. aeration) between the CV and FV did not influence any of the above parameters and resulted in similar nodule numbers and SNF activity in both treatments.

The stimulation of nodule numbers by the presence of low concentrations of ammonium has been reported previously (Gulden and Vessey, 1998). These authors showed that soybean plants fed with ammonium had significantly more nodules per plant than the ammonium-free controls. However, nitrogenase activity in the nodules of the ammonium-fed plants was 60% lower than in the controls. The results obtained in plants cultivated in the PSM, which contained low concentrations of water soluble N at the time of sowing, therefore support the findings by Gulden and Vessey (1998). Because nitrate and ammonium tend to induce differential effects on nodulation (Waterer and Vessey, 1993), it is important to mention that 82% of the N in the PSM was ammonium with nitrate constituting the rest. Even at low concentrations, nitrate is known to cause reductions in nodule numbers in a concentration-dependent fashion, while ammonium has the opposite effect (Waterer and Vessey, 1993). The observed stimulation of nodule numbers in plants cultivated in the PSM, but the reduction in nitrogenase activity on a nodule weight basis, is therefore consistent with the known effects of ammonium on nodulation.

These results demonstrated that low concentrations of ammonium in the PSM caused an inhibition of nitrogenase activity and associated ureide synthesis. The inhibition of nitrogenase activity by the presence of mineral N is well known and may be caused by increases in the resistance to O_2 diffusion in the nodule cortex (Vessey and Waterer, 1992). Interestingly, culture in sand, which is a N-free growth medium, induced very similar effects on nodule numbers and SNF compared to culture in PSM. In terms of water-holding capacity, pure sand is not a favorable growth medium and could have easily resulted in lower nodule water content and associated SNF activity. The extreme sensitivity of nitrogenase activity to even slight water deficit is well known (Serraj et al., 1999). In this context, the occurrence of more, but less functional, nodules in plants cultured in sand and PSM could both be linked to inhibitory effects on nitrogenase activity.

In conclusion, although similar source–sink relationships were maintained in plants cultured in all the growth media, large effects on nodule numbers and SNF were observed. Shoot phenotype and physiology did not provide any insight into these belowground alterations. We have shown that the presence of water soluble N in the growth medium has large effects on nodule metabolism. The effects induced by mineral N will also

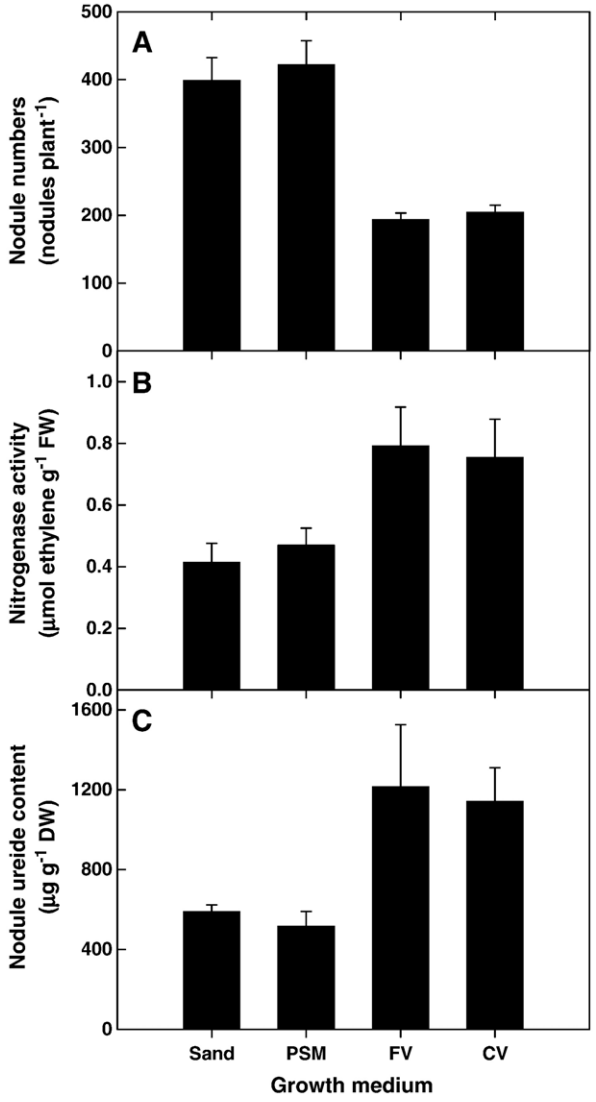

Fig. 5. Effects of growth media on nodulation and SNF in seedlings 32 days after sowing. (A) Nodule numbers per plant; (B) nitrogenase activity per nodule fresh weight; (C) ureide content per nodule dry weight. Each vertical bar represents the mean of four replicates ± standard error.

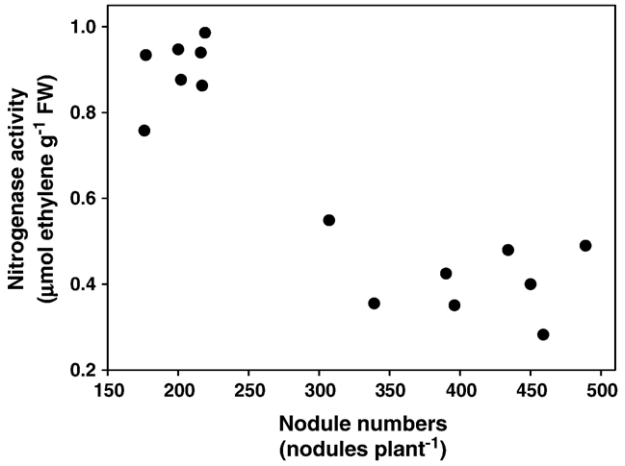

Fig. 6. Relationship between nodule numbers per plant and nitrogenase activity (on a fresh weight basis) in the same nodules. Each solid circle represents the values for these two parameters in an individual plant. The average values of these two parameters in the four different growth media treatments are shown in Fig. 5A and B.

depend on the dominant N-species (nitrate vs. ammonium) present (Waterer and Vessey, 1993), which further complicates matters. In N-free growth media on the other hand, other factors such as water holding capacity for example, also altered nodule numbers and SNF activity. It is thus clear that in studies focusing on nodule formation and nodule function, especially in the case of multi-institution collaborations, selection and standardisation of growth media for cultivating soybean plants in pots under controlled growth conditions must be regarded as a critical consideration. Because shoot growth and physiology might not be affected by the use of different growth media, effects on SNF capacity in particular might remain unnoticed. Although only a few growth media were used in these experiments, vermiculite, irrespective of particle size, appears to be suitable for cultivating soybean plants with root systems housing nodules with high SNF capacity.

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