Elemental distribution in tissue components of N₂-fixing nodules of *Psoralea pinnata* plants growing naturally in wetland and upland conditions in the Cape Fynbos of South Africa

Sheku A. Kanu · Alban D. Barnabas · Wojciech J. Przybylowicz · Jolanta Mesjasz-Przybylowicz · Felix D. Dakora

Received: 10 July 2013 / Accepted: 19 November 2013 / Published online: 24 December 2013
© The Author(s) 2013. This article is published with open access at Springerlink.com

**Abstract** There is little information on in situ distribution of nutrient elements in N₂-fixing nodules. The aim of this study was to quantify elemental distribution in tissue components of N₂-fixing nodules harvested from *Psoralea pinnata* plants grown naturally in wetland and upland conditions in the Cape Fynbos. The data obtained from particle-induced X-ray emission revealed the occurrence of 20 elements (Si, P, S, Cl, K, Ca, Ti, Mn, Fe, Ni, Cu, Zn, As, Br, Rb, Sr, Y, Zr, Mo and Ba) in nodule components. Although, in upland plants, the concentrations of S, Fe, Si, Mn and Cu showed a steady increase from the middle cortex to the medulla region of *P. pinnata* nodules, in wetland plants, only S, Fe and Mn showed an increase in concentration from the middle cortex to the bacteria-infected medulla of *P. pinnata* nodules. By contrast, the concentrations of Cl, K, Ca, Zn and Sr decreased from middle cortex to nodule medulla. The alkaline earth, alkali and transition elements Rb, Sr, Y and Zr, never before reported in N₂-fixing nodules, were found to occur in root nodules of *P. pinnata* plants grown in both wetland and upland conditions.

**Keywords** Infected/uninfected cells · Leghaemoglobin · Nitrogenase · *Psoralea pinnata* · X-ray microanalysis · Elemental maps

**Introduction**

Mineral nutrients are important for growth and cellular functioning of plants, microbes and their symbiotic interaction inside root nodules. Determinate N₂-fixing nodules such as those of the Phaseoleae (e.g. cowpea and soybean) are characterized by the presence of an outer cortex, middle cortex, inner cortex (or “nodule parenchyma”, Van de Wiel et al. 1990) and a central medulla region (Frazer 1942; Dakora and Atkins 1989), which itself consists of infected and uninfected interstitial cells (Kaneko and Newcomb 1987; Webb and Newcomb 1987). In N₂-fixing nodules, both the cortical and medulla components are interspersed by intercellular airspaces that serve as diffusional pathways for oxygen transport to respiring bacteroids in the infected cells (Dakora and Atkins 1989; Dakora and Atkins 1990a, 1991; Sherrier et al. 2005).

Nitrogen fixation by bacteroids in infected cells of root nodules is energetically a very expensive process, requiring at least 6 ATP molecules generated by oxidative phosphorylation per 2e⁻ transferred to N₂. Nitrogen fixation is thus an oxygen-demanding process. Paradoxically, however, oxygen is a potent inhibitor of nitrogenase activity, irreversibly inactivating both the Fe and MoFe proteins of the enzyme.
via oxidation of the metal-S centres (Robson and Postgate 1980) and by repression of nitrogenase synthesis (Shaw 1983). To avoid denaturation of nitrogenase enzyme, leghaemoglobin (Lb) mediates oxygen delivery at low concentrations to bacteroids inside N₂-fixing nodules (Appleby 1969; Appleby 1984; Appleby 1992; Limpens et al. 2003; Ott et al. 2005; Jones et al. 2007). This oxygen-binding protein consists of a porphyrin moiety and heme (Fe) synthesized by bacteroids (Cutting and Schulman 1971; Godfrey and Dilworth, 1971; Dénarié et al. 1976). The Lb protein is localized in the cytoplasm and nuclei of both bacteria-infected and uninfected interstitial cells (VandenBosch and Newcomb 1988; Vivo et al. 1989), with four times more Lb concentration in the infected cells relative to uninfected cells (VandenBosch and Newcomb 1988), and more Fe in the cell cytosol compared with the peribacteroid membrane (Dart and Chandler 1971). About 20–25 % of Lb in N₂-fixing nodules is oxygenated (Appleby 1984), and it is the oxidation/reduction reactions (ferrous to ferric) of Lb that delivers a free oxygen concentration of about 10 nM to respiring bacteroids in the infected cells (Appleby 1984).

Nodule formation and functioning in symbiotic legumes therefore has a heavy demand on mineral elements for both plant and bacterial growth, and metabolic functioning such as the synthesis of macromolecules. It is thus not surprising that a number of studies (Rennie and Debutz, 1986; George et al. 1993; Sparrow et al. 1995; Jensen 1997; Unkovich and Pate 2000) have established a higher root uptake and tissue accumulation of mineral nutrients by nodulated legumes when compared with non-N₂-fixing species. For example, apart from their requirement for plant and bacterial growth, nutrient elements such as P is needed in extra concentrations for ATP synthesis in support of nitrogenase activity in root nodules, just as extra Fe is required for Lb biosynthesis and the formation of nitrogenase enzyme in nodules. Although a number of studies (Atkins et al. 1984; Singleton and van Kessel 1987; Johnson et al. 2001) have addressed the role of mineral nutrients in symbiotic establishment and nodule functioning, few have examined their distribution in components of N₂-fixing nodules, especially in relation to nutritional physiology and tissue mineral metabolism.

Even though the metabolic roles of various minerals remain speculative, the occurrence of some nutrient elements has been closely associated with specific components of N₂-fixing nodules. For example, a low concentration of Mg, S and Ca was found in the inner cortex of soybean nodules formed by Bradyrhizobium japonicum strain RCR3442 when compared to strain RCR3407 (Minchin et al. 1994). In another study, P distribution was high in the bacteria-infected region, while K and Cl⁻ were lower in the same component (Mizukoshi et al. 1995). Fernandez-Paschual et al. (1996) also found a low distribution of Cl⁻ in the bacteria-infected zone when compared to the cortex. However, Ca was higher in the outer and inner cortex, but lower in the medulla, of soybean nodules (Mizukoshi et al. 1995). Furthermore, rare elements have been found in tissues of many plants (including legumes), but their functions remain unknown (Tyler 2004; Kastoril et al. 2010).

Psoralea pinnata (L.) is a legume that is adapted to both wetland and upland conditions in the Cape Fynbos of South Africa. It forms effective root nodules in the two differing habitats and derives about 60–88 % of its N nutrition from symbiotic fixation (Kanu and Dakora 2012). Psoralea is a member of the tribe Psoraleeae, which is closely related to the tribes Phaseoleae and Desmodieae (Sprent 2009), and exports ureides as the product of N₂ fixation (Kanu and Dakora 2012). The adaptation of P. pinnata to the two contrasting environments (i.e. low pO₂ in wetland vs. ambient pO₂ in well-drained upland soils) is intriguing. In this study, particle-induced X-ray emission (PIXE) and backscattering spectroscopy (BS) was used to assess and quantify elemental distribution in different nodule components (i.e. outer cortex, middle cortex, inner cortex and bacteria-infected medulla; see Fig. 1), as well as in the infected and uninfected interstitial cells of the medulla in N₂-fixing nodules harvested from P. pinnata (L.) plants growing under wetland and upland conditions in the Cape Fynbos of South Africa. (see Table 1 for soil properties).

Materials and methods

Plant material

P. pinnata (L.) plants were harvested from both wetland and well-drained upland conditions in Kleinmond and inside the...
Psoralea pinnata (L.) rhizosphere soils collected from wetland (Betty’s Bay) and dry-upland (Kleinmond) conditions in the Fynbos of South Africa

| Soil property | Wetland (mg/kg) | Upland (mg/kg) |
|---------------|----------------|----------------|
| Ca            | 253.5±53.3a    | 653.5±199.2a   |
| Mg            | 147.0±34.5a    | 49.3±12.4b     |
| K             | 40.8±1.9a      | 13.3±1.1b      |
| Na            | 55.8±10.0a     | 15.5±2.7b      |
| P             | 14.5±2.9a      | 9.3±1.9a       |
| Cu            | 0.5±0.2a       | 0.4±0.1a       |
| Zn            | 1.4±0.5b       | 8.1±2.4a       |
| Mn            | 2.3±0.4b       | 4.69±0.9a      |
| B             | 0.07±0.01a     | 0.06±0.02a     |
| Fe            | 369.3±121.5a   | 31.7±3.7b      |
| S             | 14.6±3.5a      | 3.2±0.7b       |

Mean (±S.E.) values followed by dissimilar letters in a row are significantly different at P≤0.05. The pH (KCl) for wetland and upland soils were 3.45±0.2 and 5.60±0.4, respectively.

Elemental X-ray microanalysis

Elemental analysis was performed using the nuclear microprobe at the Materials Research Department of iThemba LABS, South Africa. A proton beam of 3.0 MeV energy and 100–400 pA current was focused to 3×3 μm² spot and raster-scanned over the section using square or rectangular scan patterns with variable sizes (up to 2.5 mm×2.5 mm) and variable number of pixels (up to 128×128). Particle-induced X-ray emission (PIXE) and proton backscattering spectrometry (BS) were used simultaneously. An external 125-μm Be absorber positioned between the PIXE Si(Li) detector and a specimen was used to shield the detector from backscattered protons and to attenuate X-rays from major light elements. Processing of PIXE data was performed using GeoPIXE II software (Ryan 2000). Quantitative elemental maps were generated using the Dynamic Analysis method. In addition, PIXE and BS spectra were extracted from regions representing nodule components by drawing contours around them. Next, average concentrations from these regions were obtained from PIXE spectra, and BS spectra were used to obtain the specimen thickness and composition of major light elements for matrix corrections. The same procedure was used for single or contiguous groups of infected and uninfected cells within the medulla. Light micrographs of nodule cross-section taken before and after PIXE (especially those of cell shapes/structures and cell arrangement) were used to define the contours of each nodule component. More detailed description of the experimental procedure and experimental setup of the nuclear microprobe can be found elsewhere (Prozesky et al. 1995; Przybylowicz et al. 1999, 2005).
Histochemical test for the presence of calcium oxalate (CaC$_2$O$_4$) in nodule cortex: sample preparation and staining

To prepare samples for staining, thin hand-cut sections of freshly harvested nodules from $P.$ $pinnata$ were embedded in Technovit 7100 (a hydroxyethyl-methacrylate) according to the manufacturer’s instructions (Kulzer and Co, Wehrheim, Germany) and allowed to cure at room temperature. Semi-thin sections (4–6 μm) were cut from the embedded nodule tissue using a Reichert Ultracut S ultramicrotome system (Reichert-Jung, Austria) fitted with a glass knife, and stained for the detection of calcium oxalate following the procedure of Yasue (1969). Stained sections were examined with a Zeiss Axiocam microscope and photographed.

Statistical analysis

Element concentrations in nodule components, and in infected and uninfected cells of the medulla, were compared using 1-Way ANOVA, while elemental distribution in components of nodules developed under wetland and upland conditions were compared using 2-Way ANOVA and Duncan test ($P<0.05$, Statistica v. 8, StatSoft, USA).

Results

Elemental distribution in components of N$_2$-fixing nodules from $P.$ $pinnata$

A total of 20 elements (Si, P, S, Cl, K, Ca, Ti, Mn, Fe, Ni, Cu, Zn, As, Br, Rb, Sr, Y, Zr, Mo and Ba) were detected in symbiotic nodules from $P.$ $pinnata$ plants growing in well-drained upland soils (Table 2). This is the first report on alkali and rare earth elements (Rb, Sr, Y and Zr) being found in N$_2$-fixing root nodules of the Leguminosae. With the exception of As and Y, the concentrations of all other elements differed significantly across the nodule components (Table 2). The concentrations of P, K, S, Fe, Si, Mn, Cu and Mo were numerically and/or statistically greater in the bacteria-infected medulla region of upland nodules than cortical components (Table 2).

The same 20 elements were also detected in wetland nodules (Table 3). The distribution of P, S, Fe, Mo and Si was markedly higher in the bacteria-infected tissue than cortical components, with the levels of P, S, Fe and Mo generally showing an increase from the outer cortex to the medulla region in wetland nodules (Table 3). By contrast, the levels of Cl, K, Ca, Ni, Sr and Zr showed a decrease from the outer cortex through the middle cortex to the medulla region of wetland nodules (Table 3).

Table 2  Elemental distribution in root nodules of $Psoralea$ $pinnata$ (L.) harvested from dry-upland conditions

| Element | Outer cortex | Middle cortex | Inner cortex | Medulla |
|---------|--------------|---------------|--------------|---------|
| P       | 900±90b      | 800±40b       | 1,300±170a   | 1,300±60a |
| Ca      | 20,000±900a  | 2,500±170b    | 800±40c      | 1,200±50bc|
| K       | 3,100±190b   | 4,600±50a     | 4,300±340a   | 4,900±59a |
| S       | 1,400±30c    | 1,200±60c     | 2,400±230b   | 3,200±380a |
| Cl      | 1,400±110a   | 900±20b       | 800±90b      | 400±40c  |
| Fe      | 56±8b        | 18±1c         | 41±6b        | 200±9a   |
| Mo      | 2.0±0.3b     | 4±1a          | 3.0±0.3ab    | 5±1a     |
| Zn      | 84±6a        | 62±2b         | 58±5b        | 36±3c    |
| Ni      | 8±1bc        | 17±3a         | 3.0±0.3c     | 13±2ab   |
| Br      | 21±2a        | 5.0±0.4b      | 4±0.3b       | 5±1b     |
| Ti      | 160±20a      | 13±1b         | 2.0±0.1b     | 10±1b    |
| Si      | 1,500±130a   | 900±90b       | 1,300±200ab  | 1,600±400a|
| Ba      | 30±9a        | 8±1b          | 10±2b        | 6±1b     |
| Mn      | -            | 2.0±0.2b      | 3±1b         | 26±5a    |
| Cu      | 14±1a        | 2.0±0.2b      | 5.0±0.2b     | 15±2a    |
| As      | 4.0±0.4a     | 4.0±0.4a      | 4±1a         | 4±2a     |
| Zr      | 15±2a        | 2.0±0.3b      | 4±0.3b       | 4±1b     |
| Sr      | 82±9a        | 19±1b         | 12±1b        | 16±4b    |
| Rb      | 19±2b        | 29±4a         | 26±4a        | 22±0ba   |
| Y       | 5±1a         | 3.0±0.3a      | 2.0±0.2a     | 4.0±0.4a |

Mean (± S.E.) values followed by dissimilar letters in a row are significantly different at $P<0.05$

− not detected or below detection

Comparison of elemental distribution in wetland vs. upland nodules

Although the concentrations of Si, S, Cl, Ca, Ni, Cu, As, Y, Zr and Ba were unaltered by plant growth under upland or wetland conditions, those of P, K, Mn, Fe, Rb, Sr and Mo increased in wetland nodules, while levels of Ti, Zn and Br decreased (Table 4). The cortical components also showed differences in mineral distribution. The levels of Si, S, Mn, Fe and Mo were much greater in the outer cortex of $P.$ $pinnata$ nodules, while those of Cl, K, Ca, Ti, Ni, Cu, As, Sr, Y, Zr and Ba showed an increase in the middle cortex (Table 4). After the outer cortex, the nodule medulla was the next component with greater P, S and Fe concentration (Table 4).

There was a significant habitat x nodule component interaction for P, K, Ni, Cu, Br, Zr and Mo (Table 4). As shown in Fig. 2, the concentrations of K and P were markedly greater in the cortical and medulla region of wetland nodules compared to their upland counterparts. The distribution of Cu and Zn was also greater in the middle and inner cortex of wetland nodules than upland ones, and was the same (Zr) or greater.
Table 3 Elemental distribution in wetland nodules of _Psoralea pinnata_ (L.) harvested from wetland conditions

| Element | Outer cortex | Middle cortex | Inner cortex | Medulla          |
|---------|--------------|---------------|--------------|------------------|
| P       | 1,700±30c    | 2,600±110b    | 2,400±250b   | 3,400±90a        |
| Ca      | 14,000±1200a | 800±60b       | 1,500±120b   | 1,200±120ab      |
| K       | 17,000±800a  | 17,000±1200a  | 11,000±400b  | 10,000±100b      |
| S       | 1,600±190c   | 1,200±90c     | 2,700±150b   | 4,500±170a       |
| Cl      | 1,500±210a   | 800±110b      | 600±60bc     | 300±50c          |
| Fe      | 43±4c        | 40±3c         | 160±18b      | 200±20a          |
| Mo      | 3.0±0.3b     | 2.0±0.3b      | 2.00±0.04b   | 8±1a             |
| Zn      | 39±3b        | 71±13a        | 44±6b        | 26±2b            |
| Ni      | 25±2a        | 4±1b          | 3.0±0.3b     | 3±1b             |
| Br      | 9±1a         | 3±1b          | 9±1a         | 7±1ab            |
| Ti      | 13±2a        | 1.0±0.1b      | 2.8±1.4b     | 2.0±0.4b         |
| Si      | 1,300±80b    | 1,200±100b    | 1,200±30b    | 1,600±140a       |
| Ba      | 41±5a        | 12±3c         | 26±1b        | 6±1c             |
| Mn      | 39±2a        | 23±1b         | 33±3a        | 37±3a            |
| Cu      | 5±1a         | 4.0±0.2a      | 7±1a         | 7.0±0.3a         |
| As      | 4.0±0.1a     | 2.0±0.1a      | 2.0±0.1a     | 2.0±0.1a         |
| Zr      | 7.0±0.3a     | 5.0±0.4b      | 5.0±0.4b     | 4±1b             |
| Sr      | 110±13a      | 37±3b         | 27±3b        | 22±2b            |
| Rb      | 39±1a        | 29±1b         | 33±1ab       | 32±3ab           |
| Y       | 5.0±0.3a     | 3.0±0.3b      | 5.0±0.4a     | 2.0±0.2b         |

Mean (± S.E.) values followed by dissimilar letters in a row are significantly different at P<0.05

(Cu) in the medulla region of wetland nodules (Fig. 2). The levels of Br, Zr and Cu were markedly higher in the outer cortex of upland than wetland nodules (Fig. 2). There was also a much greater concentration of Mo in the medulla of wetland than upland nodules (Fig. 2).

Mineral concentrations in infected and uninfected interstitial cells

The distribution of mineral elements in infected and uninfected interstitial cells was assessed in upland and wetland nodules, and As, Rb, Sr, Y, Zr and Mo were found to be below detection limit. A 1-Way ANOVA analysis showed no differences in the levels of K, Ca, Ti, Mn, Ni, Zn and Cu between the two cell types in both upland and wetland nodules (Table 5). There were however significant differences in the levels of Si, P, S, Cl and Fe between infected and uninfected cells of both upland and wetland nodules (Table 5). The distribution of Si, P and Fe was much greater in infected cells compared to uninfected interstitial cells of both upland and wetland nodules (Table 5; see also Figs. 3 and 4). In contrast, Cl concentration showed a higher concentration in the uninfected cells of both wetland and upland nodules when compared to infected cells. Although K, Ca, Ti, Mn, Ni, Cu and Zn were also present in both infected and uninfected cells, their concentrations were not significantly different between the two cell types in both upland and wetland nodules.

A 2-Way ANOVA analysis of minerals in infected and uninfected cells revealed marked differences in the distribution of Si, S and Fe between upland and wetland nodules. While S was lower in cells of upland nodules, by contrast, Si and Fe occurred in greater concentrations in upland nodules (Table 6). At the cellular level, the concentrations of P and Fe were significantly greater in infected cells relative to uninfected cells (Table 6). By contrast, Cl showed a much lower level in infected cells. An analysis of significant interactions revealed no differences in the concentrations of Si, P, Cl and Fe in infected cells of nodules from upland or wetland _Psoralea_ plants (Table 6). However, S concentration was higher in the infected cells of nodules from wetland plants. Although the levels of P, S, Cl and Fe were similar in uninfected interstitial cells of upland and wetland nodules, Si concentration in uninfected cells of nodules from upland plants was twice that of uninfected cells in wetland nodules (Table 6).

Histochemical detection of calcium oxalate in outer cortex of _P. pinnata_ nodules

Microscopic examination of unstained sections of Technovit-embedded nodules revealed the presence of diamond-shaped translucent spaces within the inner portion of the outer cortex (see Fig. 5). These translucent spaces stained black upon treatment with a silver nitrate–dithio–oxamide sequence (i.e. with the Yasue (1969) procedure), indicating a positive reaction for the presence of calcium oxalate crystals in the tissues. X-ray diffraction analysis identified calcium oxalate crystals as whewellite and weddelite in dry powdered samples of _P. pinnata_ nodules.

Discussion

With the legume/rhizobia symbiosis, nutrient elements play a fundamental role in both plant and bacterial metabolism; this includes the synthesis of macromolecules such as leghaemoglobin and bacterial nitrogenase for N2 fixation, and chlorophyll for host plant photosynthesis. It is therefore not surprising that the early infection events during nodule formation involve the expression of major symbiosis-related genes (including those for nutrient uptake) in both legume and bacterial partner (Wan et al. 2005; Djordjevic et al. 2003; Rolfe et al. 2003). Some nutrient uptake-related genes activated early during symbiosis include those for siderophore production, phosphate solubilization and ion transporters for phosphate, sulphate, molybdate, iron, zinc, copper and potassium acquisition (Krusell et al. 2005). The expression of these nutrient-uptake genes suggests a metabolic connection...
between mineral nutrition and symbiotic functioning in nodulated legumes, culminating in the production of ion transporters for supporting N₂ fixation with essential nutrients.

In this study, micro-PIXE analysis consistently revealed an increase in the distribution of P, S, Fe, Mo and Si in the nodule medulla and infected cells than in the cortex and uninfected interstitial cells of *P. pinnata* nodules (Tables 2, 3 and 4). This is probably not unexpected as many of these elements are components of macromolecules in bacteroids. For example, the formation of nitrogenase requires Fe and Mo for synthesis of the oxygen-sensitive Fe and MoFe proteins of this enzyme (Robson and Postgate 1980; Shaw 1983). Thus, the concentration of Fe and Mo in infected cells, and in the bacteria-infected medulla region of active N₂-fixing nodules, would be expected to be higher as those elements are required in extra amounts for the synthesis of nitrogenase enzyme. Iron is also needed for the biosynthesis of leghaemoglobin involved in facilitated oxygen diffusion to respiring bacteroids in symbiosomes (Appleby 1984; Appleby 1992; Dordase et al. 2003), and for the synthesis of ferridoxin, an electron carrier in bacteroids.

Bacteroid reactions in symbiosomes also involve various other enzymes that can affect mineral distribution in nodules. For example, ferri-chelate reductase is an enzyme that can contribute to Fe²⁺ concentration in the peribacteroid membrane (Le Vier et al. 1996). Furthermore, the bacteroids in N₂-fixing nodules also harbour hydrogenases that are either Hup⁻ (if they evolve H₂ as the end-product of N₂ fixation) or Hup⁺ (if they oxidize symbiotically-produced H₂ to yield energy; see Rainbird et al. 1983). Although it is not clear whether *P. pinnata* nodules are Hup⁺ or Hup⁻, both types of hydrogenases are reported to require Fe, S or Ni as building blocks for their subunits (Watt and Ludden 1999). So this, in part, can

### Table 4

Comparison of elemental concentrations in nodule zones of *Psoralea pinnata* (L.) harvested from upland and wetland conditions

| Treatment | Elemental concentrations (μg g DW⁻¹) |
|-----------|-----------------------------------|
| **A.** | | |
| Growth condition | Si | P | S | Cl | K | Ca | Ti | Mn | Fe | Ni |
| Wetland | 1,400±70a | 2,600±200a | 2,600±300a | 790±90a | 13,550±630a | 4,280±1590a | 5±1b | 35±2a | 110±17a | 9±3a |
| Upland | 1,500±70a | 1,100±140b | 2,200±300a | 800±90a | 4,226±190b | 7,020±2,300a | 7±1a | 3±1b | 84±16b | 6±1a |
| Nodule component | | | | | | | | | | |
| Outer cortex | 1,600±90a | 4,400±110a | 400±20c | 7,590±1,120b | 1,230±27b | 3±0b | 23±6a | 200±7a | 2±0c |
| Middle cortex | 1,400±90ab | 1,200±150c | 1,600±300c | 1,400±100a | 9,490±2,070a | 18,580±2,960a | 14±1a | 18±6b | 39±8c | 17±4a |
| Inner cortex | 1,300±100b | 1,500±300bc | 1,300±100e | 810±30b | 9,840±1,960a | 1,840±410b | 5±1b | 17±5b | 35±6c | 7±2b |
| Medulla | 1,300±80b | 1,900±300b | 2,300±260b | 630±50b | 8,170±1,270b | 950±80b | 3±1b | 18±5b | 113±14b | 3±1bc |

2-Way ANOVA (F-statistics)

| | Growth condition | 1.60 | 123.53*** | 3.13 | 0.26 | 498.87*** | 4.11 | 7.00* | 431.72*** | 9.78** | 2.09 |
| | Nodule component | 3.44* | 21.73*** | 48.24*** | 49.68*** | 8.04*** | 40.64*** | 51.54*** | 3.93* | 90.63*** | 15.03*** |
| | Growth condition x Nodule component | 2.73 | 3.02* | 0.34 | 0.10 | 12.95*** | 2.58 | 2.32 | 1.84 | 0.74 | 10.20*** |

B. Habitat

| | Cu | Zn | As | Br | Rb | Sr | Y | Zr | Ba | Mo |
| | Wetland | 6±0a | 37±5b | 3±0a | 6±1b | 36±1a | 49±9a | 5±1a | 5±0a | 27±7a | 4±1a |
| | Upland | 7±1a | 54±4a | 3±0a | 8±2a | 21±1b | 27±6b | 4±1a | 5±1a | 15±3a | 3±0b |

2-Way ANOVA (F-statistics)

| | Habitat | 3.07 | 8.01** | 0.66 | 4.18* | 66.82*** | 29.51*** | 0.65 | 0.21 | 4.07 | 11.57** |
| | Nodule component | 26.72*** | 2.20 | 2.61 | 21.46*** | 0.66 | 80.72*** | 3.37* | 26.14*** | 9.93*** | 37.60*** |
| | Habitat x Nodule component | 32.67*** | 1.07 | 0.86 | 14.13*** | 1.55 | 2.32 | 1.37 | 13.24*** | 0.69 | 5.86** |

Values (mean±S.E.) with dissimilar letters in the same columns are significant at ***P≤0.001, **P≤0.01 or *P≤0.05
contribute to the observed increase in S and Fe concentration in infected cells and in the medulla region of Psoralea root nodules. Elemental S is also required for cellular construction of the metal S-centres of nitrogenase enzyme (Robson and Postgate 1980; Shaw 1983). So, the higher concentration of S in infected cells and in the bacteria-infected medulla region should be expected as extra amounts of S is needed for nitrogenase synthesis. However, the higher level of S in infected cells of wetland nodules relative to upland nodules (Table 3) can be attributed to the very low concentration of Si in upland soils (Table 1).

Silicon is another mineral element that has been found to promote nodule formation and symbiotic functioning in cowpea (Nelwamondo and Dakora 1999). In that study, there was a Si-induced increase in the number of bacteroids and symbiosomes in infected cells, which increased N₂ fixation (Nelwamondo et al. 2001). So, the higher concentration of Si found in infected cells and in the medulla region of Psoralea nodules in this study (Tables 2, 3 and 4) directly confirms its role in symbiotic functioning.

The equally high concentration of P in infected cells and in the medulla of Psoralea nodules (Tables 2, 3 and 4) could
reflect high rates of oxidative phosphorylation in bacteroids, a process that produces energy in the form of adenosine triphosphate (ATP) for nitrogenase activity. At the cellular level, the products of ATP hydrolysis during N₂ fixation are adenosine diphosphate (ADP), adenosine monophosphate (AMP) and inorganic P (Pi). These metabolic products (ADP, AMP and Pi) together with unhydrolyzed ATP would be expected to constitute a significant P pool in the cytosol, mitochondria and N₂-fixing bacteroids of each infected cell (Wei et al. 2004). These can together cause an increase in P accumulation in infected cells and in the bacteria-infected medulla region of symbiotic nodules, as observed in this study (Tables 2, 3 and 4). In fact, the pool size of adenylates (ATP, ADP and AMP) is quite substantial in infected cells, ranging from 45 % in bacteroids to 54 % in both cytosol and mitochondria of infected cells (Wei et al. 2004). Furthermore, these adenylate metabolites have been suggested to act as signals controlling oxygen diffusion in legume root nodules (Wei et al. 2004), operationally aided by the accumulation of K, Ca and P ions in the medulla and inner cortex (or nodule parenchyma) of N₂-fixing nodules (Minchin et al. 1995). Assuming that is true, the concentration of K, Ca and P would be expected to be high in the medulla and nodule parenchyma, as found in this study (Tables 2, 3 and 4).

Nod factor-induced Cl⁻ efflux during root hair deformation (Felle et al. 1998) is the only known function of Cl⁻ in the legume symbiosis. Yet, in this study, Cl⁻ showed a significantly high concentration in uninfected interstitial cells.
relative to infected cells (Table 3), and exhibited higher concentration in the nodule outer cortex relative to the medulla, middle or inner cortex (Tables 2, 3 and 4). Elements such as Ti, Sr, Zr and Ba also showed greater accumulation in the nodule outer cortex than the medulla and/or middle/inner cortical regions (Tables 2 and 3). Because this is the first report on the presence of Ti, Rb, Sr, Y and Zr in tissue components of N2-fixing nodules, their functions are still unknown. However, Sr2+ is reported to replace Ca2+ in supporting normal cell growth in symbiotic rhizobia (Humphrey and Vincent 1962).

The presence of Ca2+ transporters in symbiosome membrane, the accumulation of Ca2+ and nodule-specific calmodulin-like proteins in the symbiosome space during nodule functioning (Krylova et al. 2002; Liu et al. 2006), and the fact that nitrogenase activity decreased with Ca2+ depletion in symbiosomes (Krylova et al. 2002) should together suggest greater Ca concentration in the medulla and infected cells of actively-fixing nodules. That was however not the case in this study, greater Ca distribution was found in the cortical region, especially in the outer cortex (Tables 2 and 3).

This increase in cortical Ca was due to the presence of Ca oxalate crystals in the outer cortex (Fig. 5). Other studies (Sutherland and Sprent 1984) have also reported the presence of Ca oxalate in the outer cortex of nodules harvested from Phaseolus vulgaris, Glycine max, Vigna mungo, Cajanus cajan and Vigna radiata (all ureide-producing legumes), but not in root nodules of Vicia faba, Pisum sativum, Lupinus albus and Ononis repens (all amide producers). Because

| Treatment | Si   | P    | S    | Cl   | Fe   |
|-----------|------|------|------|------|------|
| Habitat   |      |      |      |      |      |
| Upland    | 1,360±170a | 2,150±240a | 1,700±200b | 560±100a | 180±20a |
| Wetland   | 900±200b  | 2,480±300a | 2,500±260a | 420±49a  | 140±12b  |
| Cell type |      |      |      |      |      |
| Infected  | 1,140±200a | 2,930±260a | 2,210±290a | 340±32b  | 190±19a  |
| Uninfected| 1,120±200a | 1,710±210b | 1,920±180a | 630±60a  | 140±13b  |
| Habitat×cell type |      |      |      |      |      |
| Infected  | 1,060±130a | 2,710±200a | 1,380±200b | 400±52a  | 210±15a  |
| Wetland   | 1,230±290a | 3,140±500a | 3,030±410a | 290±21a  | 170±17a  |
| Uninfected| 1,660±280a | 1,600±380a | 1,930±300a | 710±180a | 160±20a  |

2-Way ANOVA (F-statistics)

| Habitat | 4.37* | 0.92  | 7.74** | 1.53  | 5.34* |
| Cell type | 0.01  | 12.76** | 0.98  | 7.12* | 9.62** |
| Habitat×cell type | 8.03** | 0.10  | 8.31** | 0.04  | 0.07  |

Values (mean±S.E.) followed by dissimilar letters in a row are significantly different at $P<0.05$

Fig. 5 Light micrographs of sections of Technovit-embedded upland Psoralea pinnata (L.) root nodules showing the presence of calcium oxalate crystals (arrow) in the outer cortex next to the middle cortex cell boundary. Inner cortex (IC), middle cortex (MC), vascular bundle (VB) and outer cortex (OC) shown.
Psoralea species export ureides as the product of N₂ fixation (Kanu and Dakora 2012) and also have Ca oxalate crystals in their nodule cortex (Fig. 5), the presence of Ca oxalate in symbiotic nodules could serve as a taxonomic tool for classifying members of the Phaseoleae.

Whatever the mechanisms of mineral uptake, differences in soil properties appeared to have played a role in the observed distribution of nutrient elements in Psoralea nodules. For example, the elements P, K, S and Fe, which occurred in higher concentrations in wetland than upland soils (Table 1), also accumulated in greater levels in tissue components of nodules collected from wetland soils (Tables 2 and 3; Fig. 2). The concentrations of K and P, in particular, were about two-to threefold higher in the outer, middle and inner cortex, as well as in the bacteria-infected medulla region of wetland nodules (see Fig. 2). Thus, the consistently higher distribution of P, K, S and Fe in nodule components under wetland conditions (Tables 2 and 3) could be attributed to the greater availability of these elements in wetland than upland soil (Table 1). Conversely, the high concentration of Ca in upland soil (Table 1) could account for its increased distribution in tissue components of upland nodules (Tables 2 and 3).

Taken together, (a) the greater distribution of mineral elements such as Si, P, S and Fe in infected cells has confirmed their known roles in nodule function, (b) the alkaline earth, alkali and transition elements (Rb, Sr, Y and Zr), never reported before in N₂-fixing nodules, were for the first time found in root nodules of P. pinnata and (c) Cl⁻ (with an unknown function in root nodules) occurred in markedly high concentrations in the uninfected interstitial cells of P. pinnata nodules. With the use of genomic tools, it should be possible to determine the role of Rb, Sr, Y, Zr and Cl⁻ in nodule formation and functioning in symbiotic legumes. Hopefully, these findings would become useful only after detailed description of gene expression and metabolic pathways have been done on the effect of the various elements on growth and nodule development. Experiments with qRT-PCR on targeted genes that codify nicotianamine synthase, for example, could unravel why Fe is accumulated and/or preferentially absorbed and transported to infected cells.

Acknowledgements FDD is grateful to the South African Research Chair in Agrochemistry and Plant Symbioses, the National Research Foundation and the Tshwane University of Technology (TUT) for continued support of his research. This study forms part of a PhD degree undertaken at TUT, Pretoria. Special thanks are due to the staff of the Harold Porter Botanical Gardens in Betty’s Bay, Western Cape. X-ray diffraction analysis were performed by Remy Bucher at Materials Research Department of iThemba LABS, South Africa. Partial support by the International Atomic Energy agency (IAEA) is acknowledged.

Conflict of interest None

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.
Minchin FR, Newcomb EH (1987) Cytochemical localization of uricase and catalase in developing root nodules of soybean. Protoplasma 140:1–12

Kanu SA, Dakora FD (2012) Symbiotic nitrogen contribution and biodiversity of root-nodule bacteria nodulating Psoralea species in the Cape Fynbos of South Africa. Soil Biol Biochem 54:68–76

Kastoril RR, Maksimović IV, Zeremski-Šyttrium and higher plants*. Proc. Nat. Sci. Matica Srpska Novi Sad, F118: 87–98, UDC 661.864.1:582.3/9DOI

Krusell L, Krause K, Ott T, Desbrosses G, Kramer U, Sato S, Nakamura Y, Tabata S, James EK, Sandal N, Stougaard J, Kawaguchi M, Miyamoto A, Suganuma N, Udvardi MK (2005) The sulphate transporter SST1 is crucial for symbiotic nitrogen fixation in Lotus japonicus root nodules. Plant Cell 17(5):1625–1636

Krylova VV, Andreev IM, Andreeva IN, Dubrovo PN, Kozharinova GM, Izmailov SF (2002) Verapamil-sensitive calcium transporter in the peribacteroid membrane from Vicia faba root nodules. Russ J Plant Physiol 49:746–753

Limpens E, Franken C, Smit P, Willems J, Bisseling T, Geurts R (2003) Mechanism for short-term (15 min) changes in O2 diffusion. Ann Bot 74:613–625

Minchin FR, Iannetta PPM, James EK, Sprent JI, Thomas BJ, Witty JF (1994) Ion distribution across the cortex of soybean root nodules traced by tunsgate and 15NO3−. Soil Sci Plant Nutr 41:753–763

Minchin FR, Thomas BJ, Mytton LR (1994) Ion distribution in symbiotic nodules. New Phytol 127:56:604–617

Mizukoshi K, Nishiwaki T, Ohtake N, Minagawa R, Ikarashi T, Ohyama Y, Tabata S, James EK, Sandal N, Stougaard J, Kawaguchi M, Y , Tabata S, James EK, Sandal N, Stougaard J, Kawaguchi M, Miyamoto A, Suganuma N, Udvardi MK (2005) The sulphate transporter SST1 is crucial for symbiotic nitrogen fixation in Lotus japonicus root nodules. Plant Cell 17(5):1625–1636

Miyamoto A, Suganuma N, Udvardi MK (2005) The sulphate transporter SST1 is crucial for symbiotic nitrogen fixation in Lotus japonicus root nodules. Plant Cell 17(5):1625–1636

Nelwamondo A, Jaffer M, Dakora FD (2001) Subcellular organization of N2-fixing nodules of cowpea (Vigna unguiculata L. Walp.) genotypes grown in mixed culture and alkaline phosphate activity in roots and rhizosphere of cowpea. Plant Soil 267:191–206

Nelwamondo A, Jaffer M, Dakora FD (2001) Subcellular organization of N2-fixing nodules of cowpea (Vigna unguiculata L. Walp.) genotypes grown in mixed culture and alkaline phosphate activity in roots and rhizosphere of cowpea. Plant Soil 267:191–206

Nelwamondo A, Dakora FD (2010) Elevated level of acid and alkaline phosphate activity in roots and rhizosphere of cowpea (Vigna unguiculata L. Walp.) genotypes grown in mixed culture and at different densities with sorghum (Sorghum bicolor L.). Crop Pasture Sci 61:1–8

Ott T, van Dongen JT, Günther C, Krussell L, Desbrosses G, Vigeolas H, Samac DA, Ivashuta S, Fedorova M, Matsumoto P, Gantt JS, Vance CP (2006) Recruitment of novel calcium-binding proteins for root nodule symbiosis in Medicago truncatula. Plant Physiol 141:167–177

Przybylowicz WJ, Mesjasz-Przybylowicz J, Migula P, Nakonieczny M, Augustyniak M, Tamawska M, Tourna K, Ryszka P, Zubek S, Glowacka E (2005) Micro-PIXE in ecophysiology. X-Ray Spectrom 34:285–289

Przybylowicz WJ, Mesjasz-Przybylowicz J, Pineda CA, Churms CL, Springhorn KA, Prozesky VM (1999) Biological applications of NAC nuclear microprobe. X-Ray Spectrom 28:237–308

Ranbird RM, Atkins CA, Pate JS, Sanford P (1983) Significance of hydrogen evolution in the carbon and nitrogen economy of nodulated cowpea. Plant Physiol 71:122–127

Rennie R, Debutz S (1986) Nitrogen-15 determined dinitrogen fixation in field-grown chickpea, lentil, faba bean and field pea. Agronomy J 78:654–660

Robson RL, Postgate JR (1980) Oxygen and hydrogen in biological nitrogen fixation. Ann Rev Microbiol 34:183–207

Rolle BG, Mathiesius U, Djordjevic M, Weinman J, Hocart C, Weiller G, Bauer WD (2003) Proteomic analysis of legume-microbe interactions. Comp Funct Genom 4:225–228

Ryan CG (2000) Quantitative trace element imaging using PIXE and the nuclear microprobe. Inter J Imag Sys Tech 11:219–230

Shaw BD (1983) Non-coordinate regulation of Rhizobium nitrogenase synthesis by oxygen: Studies with bacterioids from nodulated Lupinus angustifolius. J Gen Microbiol 129:849–857

Sherrier DJ, Taylor GS, Silverstein KA, Kaneko Y, Newcomb EH (1987) Cellular compartmentation of ureide transport pathway into soybean (Glycine max) root nodules. EMBO J 6:556–560

Singleton PW, Van Kessel C (1987) Effect of localized nitrogen availability to soybean half-root systems on photosynthetic partitioning to roots and nodules. Plant Physiol 83:552–556

Sparrrow SD, Cochran VL, Sparrow EB (1995) Dinitrogen fixation by seven legume crops in Alaska. Agronomy J 87:34–41

Sprent JI (2009) Legume Nodulation: a global perspective. Wiley-Blackwell, Oxford

Sutherland JM, Sprent JI (1984) Calcium-oxalate crystals and crystal cells in determinate root nodules of legumes. Planta 161:193–200

Tyler G (2004) Rare earth elements in soil and plant systems – A review. Plant Soil 267:191–206

Unkovich MJ, Pate JS (2000) An appraisal of recent field measurements of symbiotic N2 fixation by annual legumes. Field Crop Res 65:211–228

VandenBosch KA, Newcomb EH (1988) The occurrence of leghaemoglobin protein in the uninfected interstitial cells of soybean root nodules. Planta 175:442–451

Wan J, Torres M, Ganapathy A, Thelen J, DaGue BB, Mooney B, Xu D, Webb MA, Newcomb EH (1987) Cellular compartmentation of ureide biogenesis in root nodules of cowpea (Vigna unguiculata (L.) Walp.). Planta 172:162–175

Wei H, Atkins CA, Layzell DB (2004) Adenylate gradients and Ar: O2 effects on legume nodules: Mathematical models. Plant Physiol 134:801–812

Yasue T (1969) Histochemical identification of calcium oxalate. Acta Histochemica et Cytochemica 2:83–95