Nitrogen Fixation and Translocation in Sugarcane.

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Abstract

World sugarcane production is increasing rapidly as a biofuel. In some areas in Brazil, sugarcane has been grown continually over very long periods without N fertiliser inputs. Therefore, the occurrence of N fixation has been suspected. However, quantitative studies seeking to identify the N_2 fixation sites in the plant and to record the translocation of fixed N around the plant have not yet established. A $^{15}N_2$ gas tracer experiment was conducted using young sugarcane plants to investigate the sites of N_2 fixation and also to explore the possibility of translocation of the fixed N among the plant's major organs. Young sugarcane plants (*Saccharum officinarum* L.) about 40 cm high and some 14 days after sprouting from a stem cutting were exposed to $^{15}N_2$ labeled air in a 500 mL plastic cylinder for 7 days. Following the 7-day $^{15}N_2$ feeding, some plants were potted and grown on in normal air for a further chase period. The incorporation of ^{15}N into the shoot, roots, and stem cutting was analysed at day-3, and day-7 of the labeling period and at day-14, and day-21 during the chase period. After 3 days of $^{15}N_2$ feeding, the % of N derived from the ^{15}N labeled air in the shoot, roots and stem cutting were 0.027%, 2.22 % and 0.271 %, respectively. The roots showed the highest N fixing activity followed by the stem cutting, while the incorporation of ^{15}N into the shoot was very low. After 21 days about a half of the N originating in the stem cutting had been transported to the shoot and the roots. However, the ^{15}N fixed either in the roots or in the stem cutting remained in the original parts and was not appreciably transported to the shoot.

Introduction

Sugarcane (*Saccharum* spp.) is a tall, perennial grass (family Poaceae, subfamily Panicoide), and it is cultivated in tropical and warm-temperate regions between 35°N and 35°S and from sea level to altitudes of 1,000 m on a wide diversity of soil types[1]. For many years, sugarcane has been used mainly for sugar and (much less so) for alcohol production. The use of sugarcane alcohol (ethanol) as an automotive fuel to replace gasoline has shown recent, rapid increase[2,3]. In 2006, world production of sugarcane was 1,392 million tons. This is much greater than for the other major crops such as maize (695 million tons), paddy rice (635 million tons), wheat (606 million tons) and potatoes (315 million tons)[4]. Sugarcane production is highest in Brazil (455 million tons), followed by India (281 million tons). In 2006, sugarcane was cropped over an area of 20,398,000 hectares, and the average yield was 68 tons per hectare. Sugarcane is a C4 plant, which has an efficient photosynthetic system, and it can convert up to 2% of incident solar energy into biomass. It grows up to 4 m in height and the thick stem stores a high concentration of sucrose which is present in the expressed juice at between 12 and 20% (W/V).

In Brazil, sugarcane crops accumulate between 100 and 200 kg N per hectare per year, whereas N fertilisation rates are relatively low - usually less than 60 kg N per hectare[1]. Also the response of sugarcane crops to applied N fertilisers is usually very weak or null[1,3]. In some areas in Brazil, sugarcane has been grown continuously for more than 100 years without any N fertiliser being applied at all[5]. These circumstantial evidences suggest a high potential for biological nitrogen fixation (BNF) in sugarcane. Using a ^{15}N dilution technique involving the supply of ^{15}N labeled fertiliser, Urqiaga et al.[6] calculated the contribution of BNF in several cultivars of sugarcane, and the most promising genotypes achieved about a 70% contribution. Nishiguchi et al.[7] also estimated the BNF contribution using the ^{15}N dilution technique and the results indicated that between 10% and 40% of sugarcane N was derived from BNF depending on the cultivar and also on mineral N availability. Yoneyama et al.[8] surveyed the contribution of BNF in sugarcane by a ^{15}N natural-abundance method in Brazil, the Philippines and Japan comparing ^{15}N abundance of sugarcane with that of neighbouring weeds as control plants. At many sites in Brazil a contribution from BNF was indicated but not in all cases. Again, using the ^{15}N natural-abundance method Boddey et al.[9] showed that 25-60% of the N assimilated in sugarcane at various sites in Brazil was derived from BNF.

However, both the ^{15}N dilution method and the ^{15}N natural-abundance method are indirect and, as yet no direct evidence has been presented that links diazotrophic endophytes with BNF in any of the grasses, including in sugarcane[10]. In earlier studies, Ruschel et al.[11] exposed soil-grown 60 or 90 day old sugarcane plants to $^{15}N_2$ under low oxygen concentration

(pO_2=0.02) and near normal oxygen concentration (pO_2=0.16). Significant incorporation of ^{15}N was detected in roots and shoots of intact plants only under the lower of the two oxygen concentrations. The detached roots fixed $^{15}N_2$ under pO_2=0.02, but ^{15}N incorporation was not detected in the detached shoots. Based on these results, they suggested that the site of the N fixation was within the roots and not in the shoots but that the fixed N was rapidly translocated to the shoot within 40 hours. Again working with sugarcane, Sevilla et al.[12] compared plants inoculated with wild-type *Acetobacter diazotrophicus* strain PA15, with plants inoculated with the Nif $^-$ mutant of PA15, and with un-inoculated (sterile) plants. Fixation of ^{15}N could be observed only in the shoots and roots of the plants inoculated with the wild-type PA15.

As for the presence of N-fixing bacteria in sugarcane, diazotrophic bacteria belonging to the *Beijerinckia* genera have been found in high numbers in the rhizosphere (the soil volume adjacent to, and within a few millimeters of the roots) and in the rhizoplane (the soil:root interface) of sugarcane[13]. Other diazotrophs, such as *Bacillus, Azotobacter, Derxia, Enterobacter, Erwinia, Klebsiella,* and *Azospirillum,* have also been isolated from the sugarcane rhizosphere[1]. In 1988, a new species of *Acetobacter* was found inside the sugarcane stem and named *Acetobacter diazotrophicus*[14], but this was later renamed *Glconacetobacter diazotrophicus*[15]. These organisms are called “endophytes” as they live inside the host plant tissues but without eliciting any disease symptoms. *Glconacetobacter diazotrophicus* is considered to be a major diazotrophic (nitrogen fixing) bacterium and it has been isolated from leaves, stems and roots of sugarcane plants collected from a number of sites in Brazil and also in other countries[14]. This particular bacterium does not survive free in the soil, and it is thought that it is mainly transmitted in the course of propagation which is usually done from stem cuttings or ‘setts’[16]. It is known that endophytic diazotrophic bacteria colonise the intercellular spaces and vascular tissues of sugarcane organs, without triggering any disease symptoms in the plant. The signaling mechanisms are as yet poorly understood but Cavalcante et al.[17] suggested that the ethylene signaling pathway may play a role in the establishment of the association between sugarcane and endendophytic diazotrophic bacteria. However, there is little evidence so far that *G. diazotrophicus* is actually a N_2-fixing symbiont of sugarcane or that it even expresses active nitrogenase in plants[18]. In addition the colonisation sites and movement of *G. diazotrophicus* within sugarcane plants is controversial[5,18,19].

Direct evidence for N_2 fixation and for the subsequent translocation of the fixed N within the plant requires the use of $^{15}N_2$ gas as a tracer. For some time now, we have used $^{15}N_2$ to study the assimilation and translocation of fixed N in soybean nodules[20,21]. In these experiments a

water displacement method has been used to introduce the $^{15}N_2$ gas into a cylinder in which the nodulated roots of an intact plant are first enclosed. We use a similar method here for exposing sugarcane plants grown from stem cuttings to ^{15}N-labeled air. Because N fixation has been suggested as occurring in the shoot as well as in the roots, whole sugarcane plants including the roots, a length of stem cutting and a single shoot were enclosed in a 500 mL plastic cylinder for exposure to $^{15}N_2$ gas.

The sugarcane cultivar NiF-8 (*Saccharum officinarum* L.) used in our experiments is grown commercially on Miyako-jima Island, Japan. Analysing the expressed stem juice of this cultivar, Asis et al.[22] found 21 bacterial isolates which showed positive acetylene reduction activity (indicative of N fixation) and, of these, 13 were putatively characterised as *Acetobacter diazotrophicus* and 4 as *Herbaspirillum seropedicae*. The stem cuttings were 10-15 cm long and 3 cm in diameter and contained a single bud. These were cleaned prior to being incubated in plastic trays with tap water under controlled conditions (a 14-h photoperiod at 28°C, light intensity 228 μmol m^{-2} s^{-1} PPFD and a 10-h dark period at 18°C - see Figure 1**a**). After two weeks, roots and a single shoot grew out rapidly and reached a length of about 40 cm (Figure 1**b**). Both ends of the stem original cutting were then trimmed back and small plants now with just a 4-cm stem cutting length were placed in a 500 mL measuring cylinder (inner diameter 5 cm, height 35 cm). The cylinder was first filled with a mineral-N free nutrient solution. Next, the cylinder was sealed before $^{15}N_2$ labeled air ($^{15}N_2$:Ar:O_2=24:56:20, 99.4 atom% ^{15}N) was introduced from an inlet tube at the top while the displaced nutrient solution was drained through an outlet tube until only about 50 mL of nutrient solution remained as shown in Figure 2. The enclosed plants were maintained under the same controlled light and temperature conditions. In the light, the CO_2 in the cylinder was soon depleted so we monitored this depletion and injected 2.5 mL of CO_2 by hypodermic syringe each day to maintain a certain level of photosynthesis. The nutrient solution and the inside atmosphere were otherwise not changed for 7 days. Three replicate plants were harvested on day-3 and on day-7 of the $^{15}N_2$ exposure period. Other plants that had been exposed to $^{15}N_2$ gas treatment for 7 days were then planted out in 0.02 m^2 pots filled with vermiculite and maintained under the same environmental conditions (Figure 1 **c**). Three replicates of these plants were harvested at 7 days and three at 14 days after transplanting (day-14 and day-21 from the start of $^{15}N_2$ treatment). Mineral-nitrogen free nutrient solution was supplied to the pots each day. The harvested plants were separated into shoot, roots and stem cutting, and the separate parts dried in a ventilated oven at 80°C. The N concentration and ^{15}N abundance were determined by the Kjeldahl

digestion method and GC-MS (DELTA plus XP: Thermo Fisher Scientific, Bremen, Germany), respectively.

Figure 1: A photograph of sugarcane plants cultivated in (a) water, (b) nutrient solution, and (c) a vermiculite medium irrigated daily with nutrient solution.

Figure 2: Exposure of a sugarcane plant to $^{15}N_2$.
(Left) $^{15}N_2$ gas is introduced to the cylinder displacing the nutrient solution.
(Right) The plant is exposed to $^{15}N_2$ under controlled light and temperature conditions.

Figure 3 shows changes in the total N content in shoot and roots of the young plants. The N content in the shoot at day-21 was about 13 mg. This is about twice that at day-7 just after $^{15}N_2$ exposure. The N content in the roots at day-21 was about 2 mg, about 4-times that at day-7.

Figure 3: Changes with time in total N accumulation in the shoot and roots of sugarcane plants exposed to $^{15}N_2$.

Figure 4 shows the labeled N expressed as a fraction (^{15}N%) of the total N in the stem cutting, roots and shoot after 3 days of $^{15}N_2$ exposure. The ^{15}N% in the roots was highest at 2.22%, suggesting that the roots are the most active site for N fixation. The stem cutting also exhibited ^{15}N incorporation but only at about one tenth of this level (0.27%), while the shoot contained only about one hundredth as much (0.027%) - a very low activity indeed.

Figure 4: The fraction of ^{15}N (expressed as a % of total N) fixed from ^{15}N-labeled gas in the stem cutting, roots and shoot of sugarcane plants after three days of exposure (average with standard deviations).

Figure 5 shows the mass (μg) of ^{15}N in each organ. In this case, the stem cutting showed the highest value (about 30 μg^{15}N), followed by the roots (7 μg^{15}N) and shoots (2 μg^{15}N). The high value in the stem cutting is due to the much larger N content of the stem cutting in these young plants compared to that of the shoots and roots. The stem cutting, roots and shoot contained total N amounts of 14 mgN, 0.59 mgN and 7.56 mgN respectively. Due to the large amount of N in the stem cutting, the distribution of total ^{15}N mass among the organs after 7 days

of $^{15}N_2$ feeding was higher in the stem cutting (76%) than in the roots (17%) or the shoot (7%).

Figure 5: The mass of ^{15}N (µg) in the stem cutting, roots and shoot of sugarcane plants exposed to $^{15}N_2$ for three days (average with standard deviations).

These results offer the first direct evidence of active biological nitrogen fixation (BNF) in sugarcane roots. At this early stage of development, the root system was very small and so its contribution to N_2 fixation was lower than from the much-more-substantial stem cutting (Figure 5). However, in later growth stages, and especially in the field, the root system becomes much more extensive in comparison with the stem cutting. Its relatively higher biomass will likely enable it to contribute more to the plant's total N_2 fixation than the stem cutting. This is indicated in these experiments where, five months after transplanting the rooted cuttings to pots (Figure 1 c), the roots contained 85 mgN, which accounted for about 15% of the total N in the whole plant (570 mgN).

The stem cutting showed positive N_2 fixation, which may be due to endophytic diazotrophs such as *Glconacetobacter diazotrophicus*. The N fixing microorganisms in the roots may have originated from the stem cutting because the stem cutting did not have roots at the start. It is probable that the N fixing microorganisms multiplied and colonized in the intercellular space in the growing roots In contrast to the roots and stem cutting, only negligible ^{15}N incorporation was detected in the young shoot. In the early growth stages from 2 to 3 weeks after bud break, the number of N-fixing microorganisms was too low in the shoots to detect appreciable nitrogen fixation.

Figure 6 shows changes in the percentage distribution of the total N ($^{14}N+^{15}N$) in these young sugarcane plants. At three days after the start of ^{15}N treatment, the stem cutting contains 63% of total N and the roots and shoots each contain 2.6% and 34% respectively. The percentage distribution of N in the stem cutting decreased gradually to 34% at 21 days, while the percentages in the roots and the shoot increased to 9.7% and 56%, respectively at 21 days. This suggests that the stem cutting serves as a storage organ supporting the initial growth of the roots and shoot and supplementing the current supplies of carbohydrate, N and other nutrients. Figure 6 clearly shows that the storage N in the stem cutting contributes to shoot and roots growth from planting to 21 days after ^{15}N treatment (35 days after bud break).

Figure 6: Changes with time in the fraction (%) distribution of total N (^{14}N + ^{15}N) in the

stem cutting, roots and shoot of sugarcane plants exposed to $^{15}N_2$ for 7 days.

Figure 7 shows the changes in distribution of the fixed ^{15}N among stem cutting, roots and shoot. The ^{15}N distribution among these organs was relatively constant and did not change significantly from 3 to 21 days after ^{15}N exposure. On average, the stem cutting, roots and shoot account for 80%, 15% and 5% of fixed N respectively. This result is quite different from the results obtained from experiments with soybeans exposed to $^{15}N_2$. After 10 h of $^{15}N_2$ exposure, only 36% of the fixed ^{15}N remained in the nodules, while the balance was located as follows; roots 9%, stems 17%, leaves 18%, pods 10% and seeds 10%[23]. At the end of a 10-h period of $^{15}NO_3^-$ treatment in the nutrient solution, 36% of the total ^{15}N was distributed in the roots, and 0.4% in the nodules, 17% in the stem, 36% in the leaves, 5% in the pods, and 5% in the seeds. Short term $^{15}N_2$ exposure, for just 5 min showed that 97% of the fixed ^{15}N was in the 80% ethanol soluble fraction in the nodules and was distributed in the cytosol of the nodule plant cells while only 3% remained within the bacteroid, a N fixing rhizobium in the nodules[24]. In the case of soybean nodules, N_2 is fixed by the bacteroid to ammonia and the ammonia or ammonium is readily excreted to the cytosol of the plant cells. The ammonium is then assimilated within the GS/GOGAT system[20,25], and synthesised mainly to ureides, allantoin and allantic acids, and the ureides are transported around the various plant organs via the xylem[26].

Figure 7: Changes with time in the fractional distribution (%) of total ^{15}N in the stem cutting, roots and shoot of sugarcane plants exposed to $^{15}N_2$ for 7 days.

The mechanism of N transfer to the host sugarcane plant from the N-fixing endophyte has not yet been resolved. There are two possible pathways for this transfer to occur. The first is by an active excretion of fixed N from the healthy bacterium to the appoplast of host tissue and the plant cells then absorb the released N compounds, and the other is for the bacterium to fix N and to proliferate in the host and for its complement of fixed N to be released to the host cell only after its death and disintegration. No direct evidence has been obtained here, regarding this question but the data of Figure 7 suggest the second pathway. The fixed N does not seem to be readily excreted from the endophyte and so available for transport to vigorously growing organs such as the shoot and roots. In a separate experiment, sugarcane plants were harvested after cultivation for 3 months following a one-day exposure to $^{15}N_2$, enrichment with ^{15}N could not be detected in the shoots (data not shown).

The samples were extracted with 80% ethanol, and separated into ethanol-soluble and ethanol-insoluble fractions. The ethanol-soluble fraction contains low molecular weight compounds such as amino acids and ammonia, and the ethanol-insoluble fraction contains large molecules such as proteins and nucleic acids. The soluble N, as a proportion (%) of total N was relatively high in the stem (about 30%) but was much lower in the roots and shoot (5% and 7.5% respectively). The higher proportion of soluble N in the stem may indicate that this is the source organ for stored N within the plant with storage proteins being decomposed to amino acids and/or other low molecular weight compounds in readiness for translocation. In the sugarcane experiment, the abundance of ^{15}N in the soluble fractions at day-3 and at day-7 during $^{15}N_2$ exposure was lower than in the insoluble fractions (data not shown). This suggests that the fixed N is primarily incorporated into the cell components, such as protein of the endophytic bacteria, rather than being transferred to the cells of the host plant. This contrasts with soybean nodules where the abundance of ^{15}N in the soluble fraction just after $^{15}N_2$ exposure was about 5-times higher than that in the insoluble fraction[20,21]. This enhancement is due to an active turnover and to the small size of the soluble fraction in the nodules

The abundance of ^{15}N in the free amino acid pool was determined after separation by two-dimensional thin layer chromatography[27]. In the stem cutting, asparagine and alanine were the major free amino acids, while 4-amino butyric acid (GABA) and valine were also detected. In the shoot, asparagine and alanine again dominated the free amino acid pool, while 4-amino butyric acid, valine, hydroxyproline, glycine, glutamate and glutamine were also detected. After 7 days of $^{15}N_2$ exposure, the abundance of ^{15}N alanine and ^{15}N asparagine in the stem and shoot were both lower than 0.04 atom%. These very low atom% values support the interpretation that

the endopyte does not excrete low molecular weight compounds such as ammonia and amino acids. Lethbridge and Davidson[28] suggested that the endophytic bacteria only transferred fixed N to the plant when they died and were substantially mineralised. In contrast, Cohjo et al.[29] suggested that 50% of the N fixed by *Gluconacetobacter diazotrophicus* was transferred to the co-cultured yeast in an *in vitro* system, so a small portion of the fixed N may be used by the host plant tissue at the site of N fixation.

From the results obtained in this experiment with young sugarcane plants, it is confirmed that the roots are the most active site of N fixation followed by the stem cutting. The sugarcane cuttings were initially cultured in water not in soil, so the N fixing endophytes in the roots might originate from the stem cutting or root primordia. To support active N_2 fixation, nitrogen-fixing bacteria may move into the developing roots, and there colonise in the intercellular space. In an earlier study with NiF 8 sugarcane[30], after 5 months of cultivation in a pot supplied with ^{15}N labeled mineral fertilizer, it was estimated that the roots contributed higher proportions of BNF (26%) than the stem (14%) and leaves (21%). Compared with the stem and shoot, the roots offer certain advantages as N fixation sites. These are (1) that the host plant provides nutrients, not only to the root endophytes but also to the microorganisms present in the rhizosphere and rhizoplane through root exudates and from broken root tips and fine root turnover, and (2) that the oxygen concentrations in the roots and soil are usually lower than in the atmosphere (pO_2 0.21). Low pO_2 is beneficial in that it protects nitrogenase from oxygen damage, this enzyme being very sensitive to high pO_2.

Nitrogen fixation in stem cuttings of sugarcane is probably due to the activity of endophytes. Because very young sugarcane plants were used in our experiments, the finding of an extremely limited N fixation activity in the shoots should not necessarily be taken to imply an insignificant stem endophytic contribution in the N economies of mature field-grown sugarcane plants. Many sugarcane endophytes (eg. *Glconacetobacter diazotrophicus*) are adapted to conditions of high sucrose concentration while our very young shoots did not have mature stems that offered these conditions. Recently Zakria et al.[31] reported very low $^{15}N_2$ fixation activity in mature sugarcane stem pieces (of NiF-8 and Ni15) in which sugar concentrations were up to 20%. These stems showed inconsistent acetylene reduction activities, some quite high but others very low. Therefore, N fixation in mature sugarcane stems may be influenced also by the types and numbers of endopytes present, by stem age/maturity, and by other undefined physiological conditions.

The absence of significant N translocation in our experiments does not mean that there

can be no contribution of N fixation in the roots to shoot growth. When the N-fixing bacteria in the root eventually die, their decomposition products may well contribute to the growth of the roots and to other parts of the plant. If this does not occur, then the N fixed in the roots will at least contribute to soil fertility in the field after the natural processes of root turnover and decomposition. Ando et al.[32] calculated N fixation rates, fertiliser efficiency and the turnover of organic matter in sugarcane production in Thailand. Using the ^{15}N natural-abundance method, they estimated that the contribution of crop N fixation to the overall N economy of many of the plantations was about 0-30%. Meanwhile, the N contributions from applied mineral fertilisers were about 18-31%. This implies that N supplied from other soil-N sources such as from decomposing crop organic matter is important for supporting the fertility of the soil under sugarcane production. Other than in Brazil, 150-250 kg urea-N per hectare per year is usually applied to sugarcane, the actual amount depending on soil fertility, on genotype and on target yield[33]. By promoting biological N fixation through associative diazotrophs, the cost associated with N fertiliser usage in sugarcane production can be reduced and environmental problems consequent upon the use of excessive chemical fertilisers can be avoided. Further research will be important for promoting more efficient N fixation rates in sugarcane production.

METHODS SUMMARY

Sugarcane (*Saccharum officinarum* L., cultivar NiF-8) stem sections (setts) about 15 cm long and 3 cm diameter with one bud were soaked in trays filled with tap water, and grown under a 14-h photo period at 28°C (light intensity at 228 μmol m^{-2} s^{-1} PPFD) and with a 10-h dark period at 18°C in a Biophotochamber for 14 days. The roots and a single shoot grew rapidly to about 40 cm. Both ends of the stem were then trimmed back to form a small plant having a stem cutting about 4 cm long. This was placed in a 500 mL transparent measuring cylinder for 7 days. The cylinder was sealed with a rubber bung containing a gas-inlet tube and a liquid-outlet tube. The cylinder was previously filled with a mineral-N free nutrient solution. Air labeled with $^{15}N_2$ ($^{15}N_2$:Ar:O_2=24:56:20, 99.4 atom% ^{15}N) was introduced through the inlet tube displacing the nutrient solution through the outlet tube, until only about 50 mL of the nutrient solution remained. The two inlet/outlet tubes were then sealed off with pinch cocks. Each day 2.5 mL of CO_2 gas was injected to maintain a certain level of active photosynthesis. The cylinders each containing one sugarcane plant were placed in the Biophotochamber under the same temperature/light conditions for either 3 days or 7days. After $^{15}N_2$ exposure for 7 days, some

plants were transplanted to 0.02 m^2 pots containing vermiculite, and grown on in a greenhouse with daily additions of mineral-N free nutrient solution for an additional 14 days.

Plants were sampled in triplicate on four occasions. On day-3 and day-7 during $^{15}N_2$ exposure, and on day-14 days (7 days from the start of growth in the greenhouse) and day-21 (14 days from start of growth in the greenhouse). Plants were divided into three parts, shoot, roots and stem cutting. These were dried in a ventilated oven at 80°C and ground to a fine powder with a vibrating mill (CMT co. Ltd., Tokyo, Japan). Powder samples (50 mg) were extracted in ethanol. Total N was determined by Kjeldahl digestion and ^{15}N abundance was determined by gas chromatography-mass spectrometry.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Online Methods

Plant material and cutting growth

Sugarcane (*Saccharum officinarum* L., cultivar NiF-8) stem cuttings about 15 cm long and 3 cm in diameter with one bud were soaked in a tray filled with tap water and grown under a 14-h photo period at 28°C (light intensity at 228 μmol m^{-2} s^{-1} PPFD) and with a 10-h dark period at 18°C in a Biophotochamber (TAITEC LX-3000, Tokyo, Japan) for 2 weeks. The single shoot grew rapidly reaching about 40 cm.

Exposure of sugarcane plants to ^{15}N labeled air

Both ends of the stem cutting were trimmed and the resulting small sugarcane plant, now with about 4 cm of remaining stem cutting, a good number of new roots and about 40 cm of fast-growing shoot, was placed in a 500 mL plastic measuring cylinder for 7 days. The cylinder was sealed at the top with a rubber bung having both a gas-inlet tube and a water-outlet tube (reaching to the bottom of the cylinder). The cylinder was filled with mineral-nitrogen free nutrient solution. The composition of this solution was (mg L^{-1}); $CaCl_2 \cdot H_2O$ (183), $MgSO_4 \cdot 7H_2O$ (123), K_2SO_4 (109), KH_2PO_4 (8.5), KCl (0.935), $FeSO_4$ (13.9)+$EDTANa_2$ (18.6), H_3BO_3 (0.367), $MnSO_4$ (0.189), $ZnSO_4 \cdot 7H_2O$ (0.144), $CuSO_4 \cdot 5H_2O$ (0.032), $CoSO_4$ (0.028), $(NH_4)_2Mo_7O_{24}$ (0.004), $NiSO_4 \cdot 6H_2O$ (0.0035). The pH was adjusted to 6.0.

Air labeled with $^{15}N_2$ ($^{15}N_2$:Ar:O_2=24:56:20, 99.4 atom% ^{15}N, purchased from Shokotusho Co. Ltd Japan, gaseous impurities not detectable by gas chromatography) was introduced through the inlet tube and the displaced nutrient solution was drained from the outlet tube, until only about 50 mL remained. The inlet and outlet tubes were immediately sealed with pinch cocks applied to short lengths of silicon tubing. To maintain a minimum level of photosynthesis, 2.5 mL of CO_2 gas was injected each day. The solution and gas inside was otherwise not changed. After 7 days of $^{15}N_2$ exposure, some plants were transplanted to 0.02 m^2 pots filled with vermiculite, and grown on in a greenhouse for an additional two week chase period with daily irrigations with nitrogen-free nutrient solution of the same composition. Replication throughout the experiment was in triplicate.

Sampling and analysis

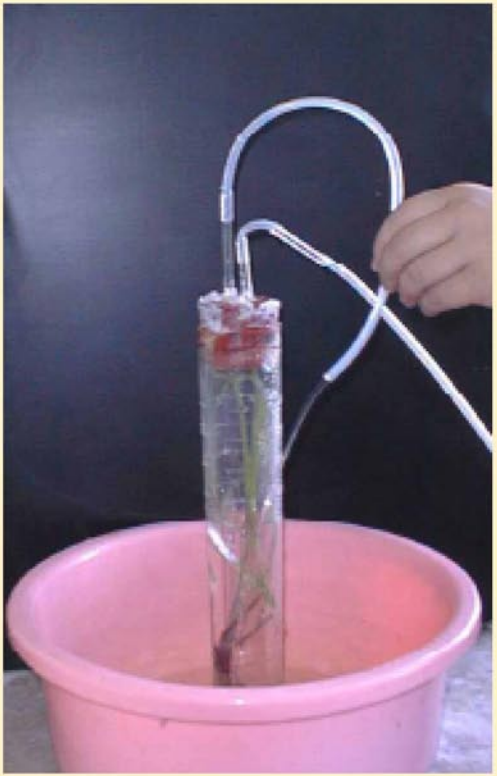
Sampling was done at four times. The first and second samples were taken at day-3 and day-7

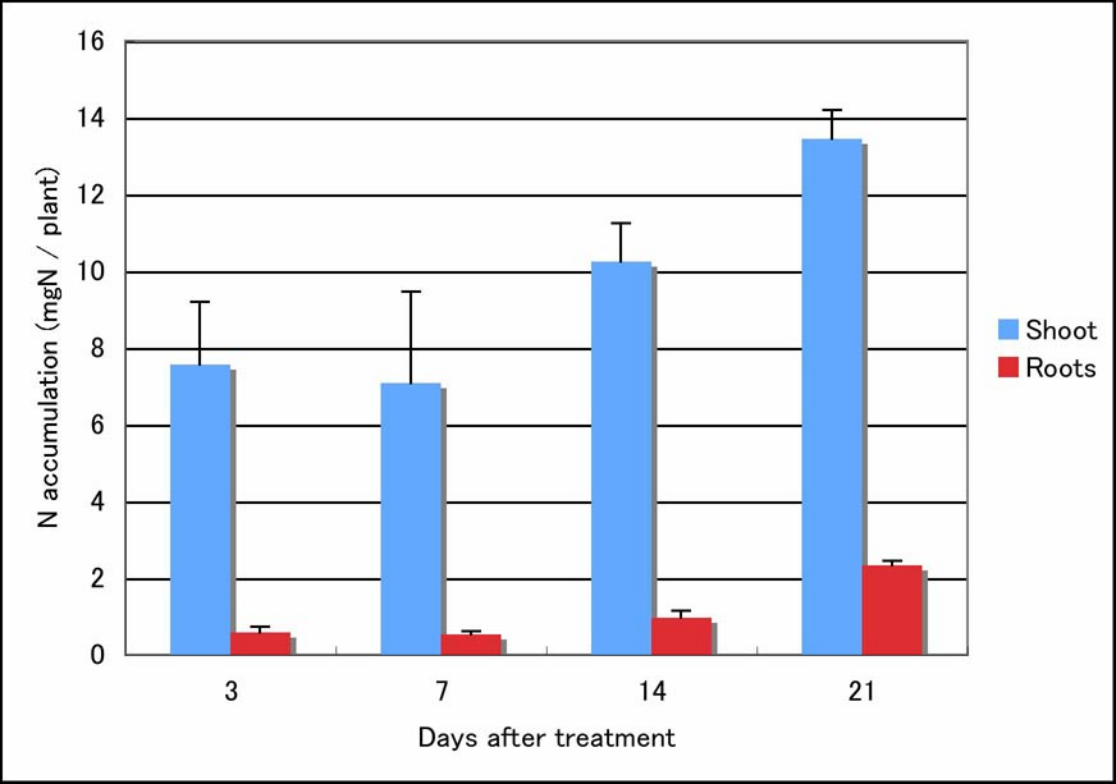
from the start of the $^{15}N_2$ exposure period. The third and fourth samples were made after transplanting to pots at day-14 (7 days after transplanting) and at day-21 days (14 days transplanting). Plants were divided into three parts, shoot, roots and stem cutting, dried in a ventilated oven at 80°C and ground with a vibrating mill (CMT Co. Ltd., Tokyo, Japan).

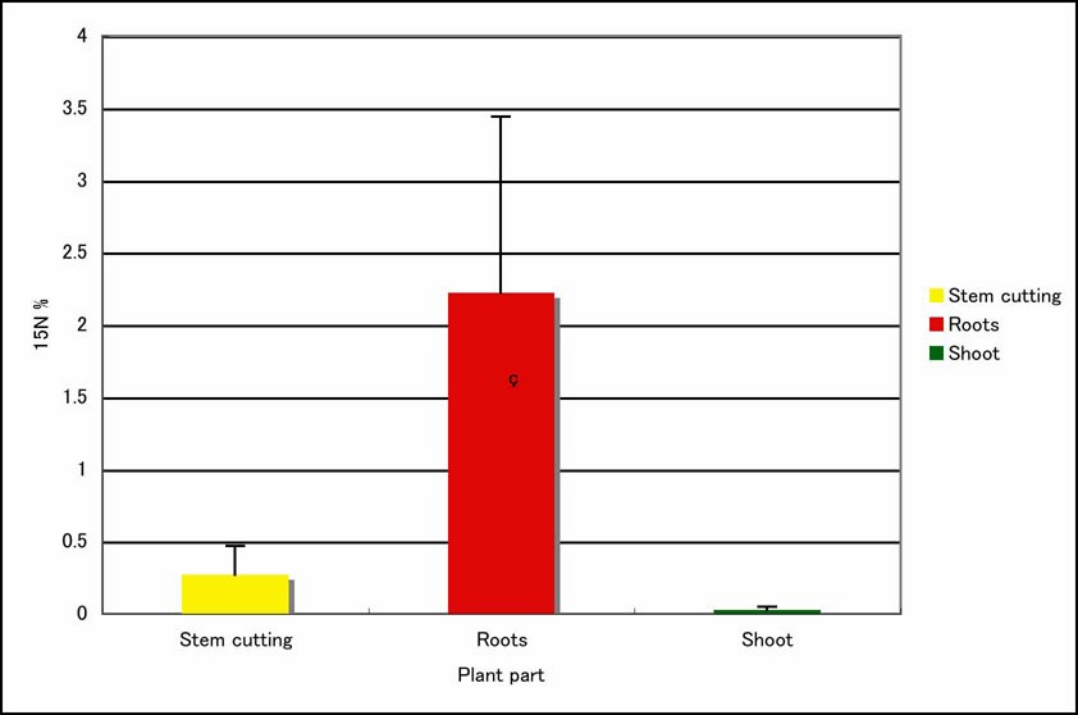
Powder samples (50 mg) were extracted in 80% ethanol and divided into residue and extract by centrifugation. Total N-content was determined by Kjeldahl digestion. The abundance of ^{15}N was determined by gas-chromatography, mass-spectrometry (GC-MS) DELTA plus XP (Thermo Fisher Scientific, Bremen, Germany).

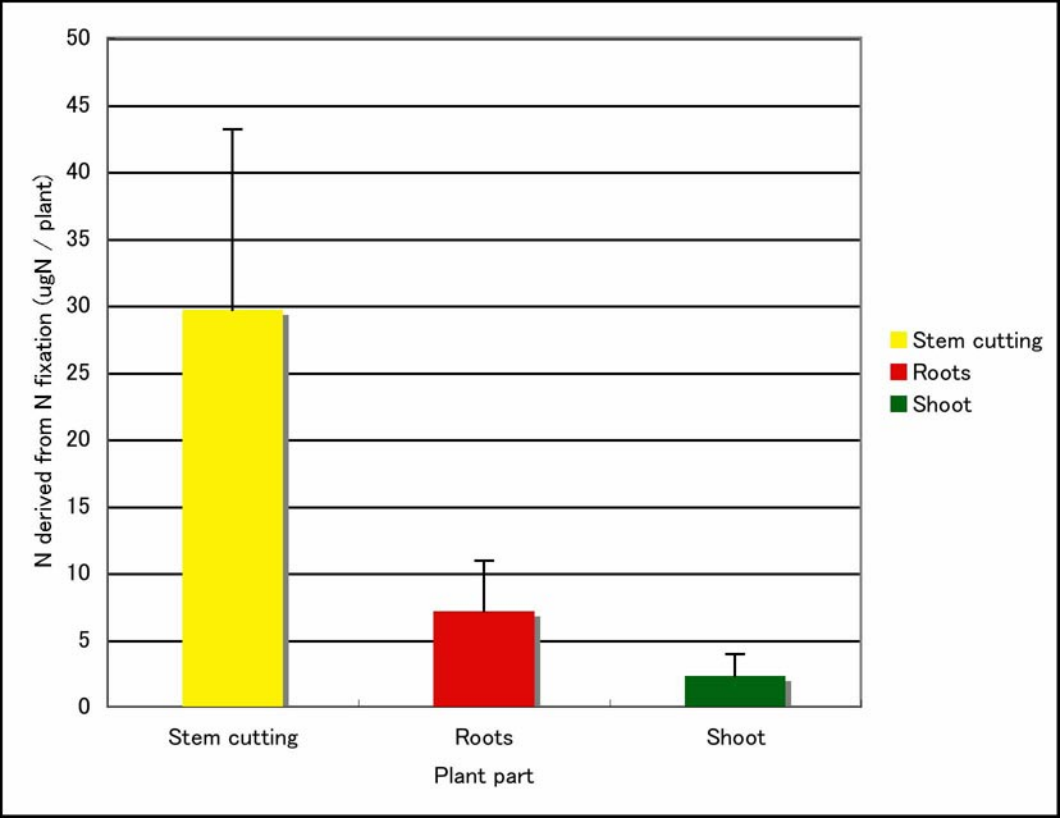
Stem cutting and shoot powder samples (100 mg) were extracted in 80% ethanol, evacuated to dryness and re-dissolved in water. The cationic fraction was recovered using a cation-exchange column (DOWEX 50 H^+ form) and separated by two dimensional silica-gel, thin-layer chromatography using phenol:water (4:1 V/V) for the first solvent and butanol:acetic acid:water (4:1:1 V/V/V) for the second solvent[27]. After spraying ninhydrin solution, each amino acid spot was collected and extracted from the silica gel using 50% ethanol, and the ^{15}N abundance was determined by emission spectrometry[27].

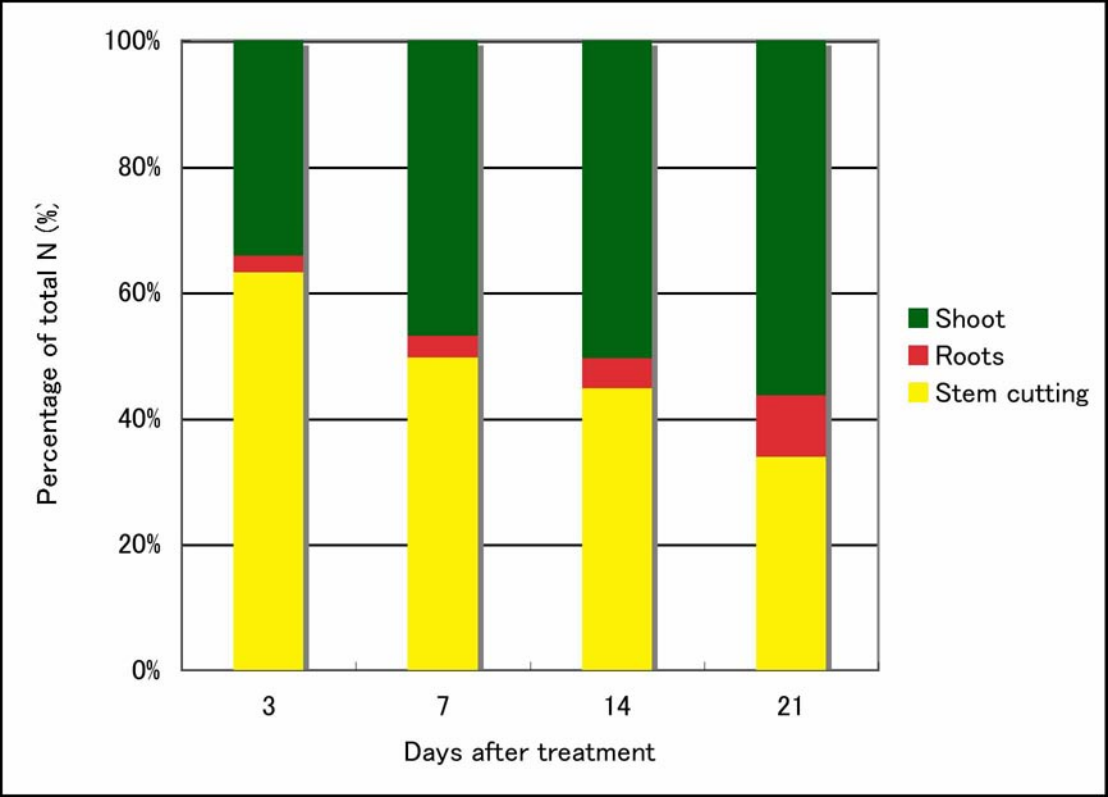

a
b
c

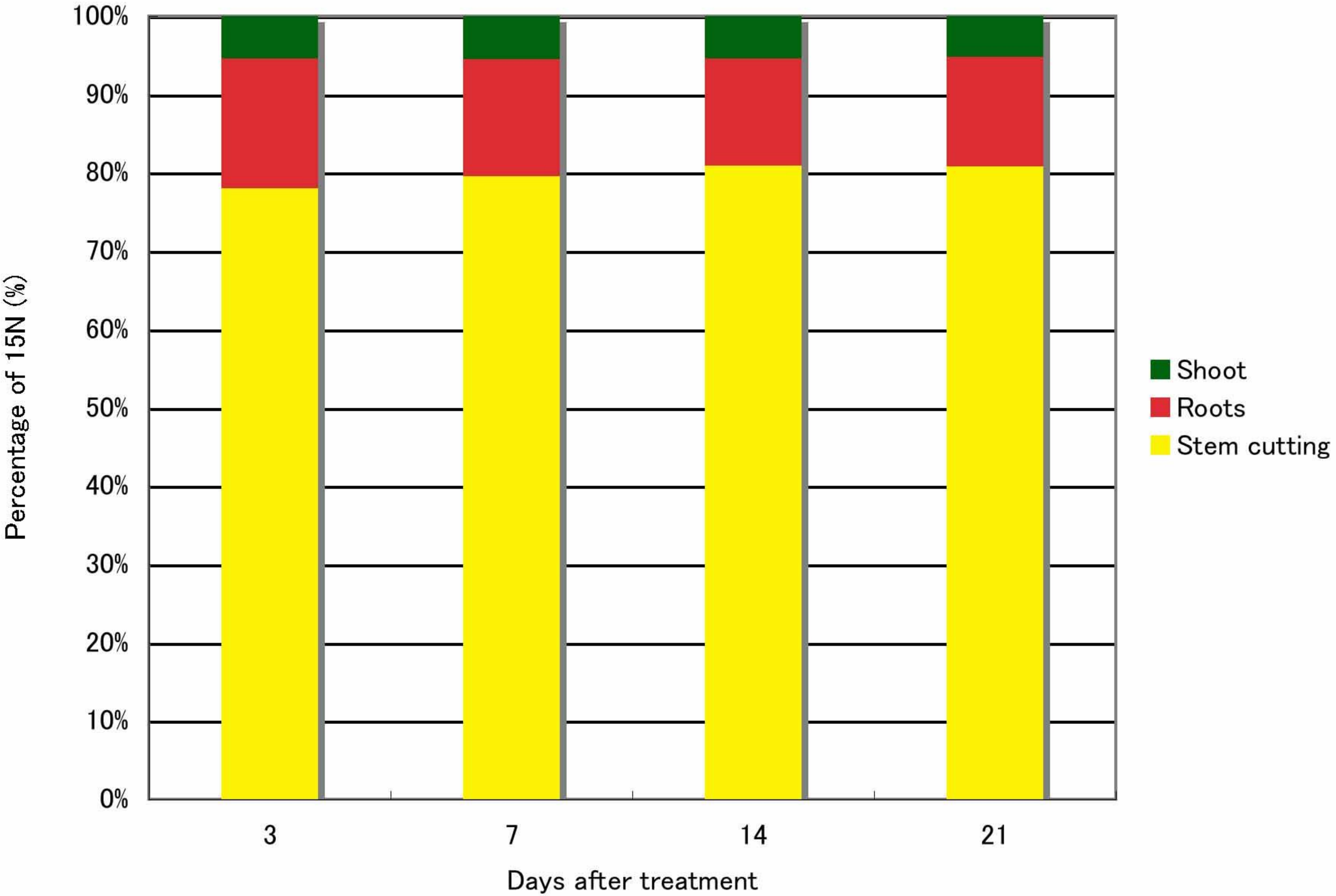



16
14
12
10
8
6
4
2
0
N accumulation (mgN / plant)
Shoot
Roots
3
7
14
21
Days after treatment


4
3.5
3
2.5
2
1.5
1
0.5
0
15N %
a
Stem cutting
Roots
Shoot
Plant part
Stem cutting
Roots
Shoot


50
45
40
35
30
25
20
15
10
5
0
N derived from N fixation (ugN / plant)
Stem cutting
Roots
Shoot
Stem cutting
Roots
Shoot
Plant part


100%
80%
60%
40%
20%
0%
Percentage of total N (%)
3
7
14
21
Days after treatment
Shoot
Roots
Stem cutting


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percentage of 15N (%)
3
7
14
21
Days after treatment
Shoot
Roots
Stem cutting