



Endophytic N₂ fixation in sweet potato: responses to N, P, and K inputs and visualization of ¹⁵N₂ utilizing bacterial cells via Raman spectroscopy

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Abstract

Fertilizer-N strongly limits non-leguminous crop yields; however, sweet potato (*Ipomoea batatas*) is an exception, likely due to its ability to acquire atmospheric N₂ via endophytic diazotrophs. Using Raman spectroscopy, we found that in ¹⁵N₂-fed sweet potato, some endophytic bacteria contained ¹⁵N, providing direct evidence of N₂ fixation *in planta*. To assess N₂-fixing capability, pot experiments were conducted by varying N, P, and K fertilizer inputs. Sweet potato showed higher N content than the non-N₂-fixing *Ipomoea aquatica*; additionally, it showed increased N content which was 1.4-fold higher than the fertilizer-N input. Its δ¹⁵N values were closer to those of N₂-fixing soybean, with an estimated 11–56% of plant N derived from N₂. The estimated amount of fixed-N in sweet potato was negligible without fertilizer-N; however, a gradual accumulation leading to an immediate saturation was observed with increasing fertilizer-N. During this state, increasing P supply linearly enhanced the capability, reaching 13 g N m⁻², comparable to that of legumes. However, K inputs affected neither N₂ fixation nor growth owing to strong K acquisition from the soil even without fertilizer-K. Our results indicate extensive N₂ fixation in sweet potato, depending on its nutritional status, particularly P; resonance Raman spectroscopy facilitates the visualization of active N₂-fixing bacteria on a single-cell scale.

Keywords Endophyte · Nitrogen fixation · Phosphate · Potassium · Resonance Raman spectroscopy · Stable isotope probing (SIP)

Introduction

Sweet potato (*Ipomoea batatas*) is well recognized in Japan as a “rescue crop” that saved people from starvation during times of famine and war. One of the reasons is that the plant can produce a substantial amount of starch in its tuber even in nutrient-poor soil, in contrast to cereals that require large amounts of fertilizer inputs, especially N. Owing to the low N requirement, it has long been speculated that sweet potato may acquire N₂ from the atmosphere (Hill et al. 1990). Yoneyama et al. (1997) compared the δ¹⁵N values of sweet potato with those of pumpkin and estimated that 26–44% of plant N in sweet potato was derived from the atmosphere. The percentage

values are equivalent to those derived from fertilizer-N of non-leguminous crops (Yamaguchi 1991; Stevenson and Cole 1999; Yan et al. 2020); this likely explains the minimal fertilizer-N requirement of sweet potato.

Endophytic bacteria of sweet potato are considered responsible for N₂ fixation (Adachi et al. 2002; Terakado-Tonooka et al. 2013; Yoneyama et al. 2017; Itoh et al. 2019). Similar to sweet potato, N₂ fixation by endophytic bacteria likely contributed considerably to plant N economy in sugarcane (Cavalcante and Döbereiner 1988; Boddey et al. 2001; Sevilla et al. 2001) and certain maize landraces (Van Deynze et al. 2018). Bacterial candidates involved in endophytic N₂ fixation have been investigated via genome analysis (Reiter et al. 2003; Terakado-Tonooka et al. 2008; Khan and Doty 2009; Shinjo et al. 2016; Yoneyama et al. 2017; Puri et al. 2018a, 2018b; Shinjo et al. 2018; Itoh et al. 2019). However, the detection of genes encoding nitrogenase does not necessarily imply that the endophytes actually fixed N₂ *in planta*. To identify functional N₂-fixing bacteria in soil, Cui et al. (2018) used resonance Raman spectroscopy with ¹⁵N₂, which enabled the

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differentiation of bacterial cells containing ^{15}N ; however, such differentiation is yet to be established *in planta*.

In leguminous plants, excess N supply inhibits nodule development and nitrogenase activity (Marschner 1995), and the inhibitory effects are not only direct but also systemic in split-root systems (Daimon and Yoshioka 2008), indicating that the N_2 fixation is likely regulated by the nutritional status. However, little is known about how nutritional status affects the ability of endophytic N_2 fixation of plants without root nodules, including sweet potato, although soil N content may affect bacterial diversity in the rhizosphere of sweet potato (Tangapo et al. 2018). In this study, we evaluated the effects of N, P, and K inputs on N_2 fixation in sweet potato. We further attempted to detect functional endophytic bacteria in sweet potato fed with $^{15}\text{N}_2$ via resonance Raman spectroscopy; this attempt would provide direct evidence for realized N_2 fixation *in planta* that cannot be addressed using genomic approaches.

Materials and Methods

Plant materials and growth conditions

In 2019, pot experiments were conducted at the Higashiyama Campus of Nagoya University, Nagoya, Japan. Each pot (24 cm diameter, 23 cm depth) was filled with 6 L of a mixture of Andosol (29% sand, 55% silt, and 16% clay; pH 5.7; total C 89 g kg^{-1} , total N 7.2 g kg^{-1}) and vermiculite (1:1). In the soil medium, N (urea, 46% N), P (superphosphate, 17.5% P_2O_5), and K (potassium chloride, 60% K_2O) fertilizers were uniformly applied at various rates (Supplementary Table S1); four levels of the N, P, or K inputs were applied, while the inputs of the other two elements were held constant. A shoot (approximately 40 cm in length) of sweet potato (*Ipomoea batatas*, cv. Beniazuma) was transplanted into each pot on May 31, 2019. On the same day, six seedlings of 10-day-old non- N_2 -fixing water spinach (*Ipomoea aquatica*, Asahi Noen Seed Co. Ltd., Japan) were also transplanted into each pot, which received P and K fertilizers at constant rates, with four levels of N fertilizer inputs same as those of sweet potato (Supplementary Table S1). All the plants were harvested 109 days after transplantation.

In 2020, pot experiments were repeated to observe the saturated growth response of sweet potato over a wider range of P input (Supplementary Table S1). Based on the results in 2019, the input of fertilizer-N was doubled, whereas K fertilizer was not applied. As an N_2 -fixing plant, soybean (*Glycine max.* cv. Fukuyutaka) seeds coated with a rhizobium inoculant (Mame-Zo, Tokachi Federation of Agricultural Cooperatives, Japan) were sown in each pot filled with the soil medium as described above; four seedlings were

established per pot. For soybean whose main N source is atmospheric N_2 , no fertilizer was applied to determine the $\delta^{15}\text{N}$ value of the plants. The growth period of soybean was 99 days from July 4.

Four replicated pots were prepared for each treatment. Each pot was irrigated from the bottom using a tray (30 cm in diameter, 4 cm in depth), whose water level was maintained at 1 cm throughout the growth period. During sampling, the shoots of the sweet potato were separated into stems and leaves. The roots and tubers were carefully washed free of soil with tap water. The samples were oven-dried (80 °C) to measure their dry weights and then ground for further analyses.

N, P, and K quantification of plant parts

The ground samples of each organ were used to determine the total N concentration using an elemental analyzer (FLASH2000, Thermo Fisher Scientific Inc., USA). To determine the $\delta^{15}\text{N}$ value in the whole plant, the ground sample from each organ was thoroughly mixed based on the weight ratio of each organ when the samples were segregated. Each of the mixed samples of a whole plant was combusted in the elemental analyzer, and a part of the combustion gases was introduced into an isotopic ratio mass spectrometer (Delta Plus, Thermo Fisher Scientific Inc. Worcester, MA, USA) to determine the $\delta^{15}\text{N}$ value. Plant P concentrations were determined colorimetrically according to Watanabe and Olsen (1965) once the ground samples of each organ were reduced to ashes at 495 °C for 2 h. To determine K concentration, the ground samples in each organ were extracted with 15 mL of 1 M HCl by shaking for 1 h and then left overnight; the solution was then passed through a filter paper (No. 6, diameter; 90 mm, ADVANTEC Inc., Japan). Aliquots of the filtrate were then analyzed using a digital flame photometer (ANA-135, Tokyo Photoelectric Co., Ltd., Japan) to determine the K concentration.

Estimation of plant N derived from fertilizer and the atmosphere

We calculated the % N derived from fertilizer (% Ndff) and from the atmosphere (% Ndfa) among the total plant N following the equations below:

$$\% \text{Ndff} = \frac{\delta^{15}\text{N}_{\text{water spinach only with soil-N}} - \delta^{15}\text{N}_{\text{plant with a mixture of fertilizer-and soil-N}}}{\delta^{15}\text{N}_{\text{water spinach only with soil-N}} - \delta^{15}\text{N}_{\text{water spinach only with fertilizer-N}}} \times 100$$

where the $\delta^{15}\text{N}$ value for water spinach only with soil-N represents the mean N of the plant without any N fertilizer input per pot, and the $\delta^{15}\text{N}$ value for water spinach only with fertilizer-N represents the mean N of the plant grown in vermiculite amended with urea.

$$\% \text{ Ndfa} = \frac{\delta^{15}\text{N}_{\text{water spinach at particular input of fertilizer-N}} - \delta^{15}\text{N}_{\text{sweet potato at particular input of fertilizer-N}}}{\delta^{15}\text{N}_{\text{water spinach at particular input of fertilizer-N}} - \delta^{15}\text{N}_{\text{soybean in unfertilized soil}}} \times \% \text{ Ndfa in soybean}$$

where the $\delta^{15}\text{N}$ value for water spinach at a particular input of fertilizer-N represents the mean value for each N input. To avoid a negative % Ndfa value, it is assigned a zero if the $\delta^{15}\text{N}$ value for sweet potato is higher than that of water spinach at a particular fertilizer-N input.

Acetylene reduction assay

The N_2 -fixing activities per biomass of each organ were investigated using an acetylene reduction assay (Hardy et al. 1968). Since the middle of June in 2021, sweet potato (*Ipomoea batatas*, cv. Beniazuma) was grown without fertilizer input in a field at Higashiyama Campus in Nagoya University with a 30-cm plant distance and 60-cm row spacing. The plants were sampled 3 months after transplantation for the acetylene reduction assay. The roots and tubers were thoroughly washed under running tap water to remove soil particles. Each plant part was packed into a glass tube (120 mL) with 3 mL of distilled water to avoid desiccation and then sealed with a silicone lid. The 10% (v/v) of the gas in the tube was replaced with acetylene and immediately incubated at 25 °C for 30 min. After 30 min, the ethylene concentration in each tube was determined using a gas chromatograph (GL Sciences Inc., Japan). After measurement, the samples from each tube were dried at 80 °C and weighed to indicate the activity per unit of dry weight.

Raman spectroscopy

A fresh shoot of sweet potato (approximately 30 cm in length) grown in the field at Higashiyama Campus in Nagoya University was cut and inserted into a pot (1 L) filled with wet vermiculite for 7 days in a growth chamber under the following conditions for root development: light intensity, 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$; relative humidity, 60%; temperature, 30 °C (day)/25 °C (night); photoperiod, 14 h (day)/10 h (night). Post root development, it was transplanted into a transparent plastic box (195 mm in length, 103 mm in width, and 20 mm in depth) filled with vermiculite. During growth, a nutrient solution (6.0 mM CaCl_2 , 3.0 mM NaNO_3 , 1.0 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 3.9 mM MgSO_4 , 20.0 mM K_2SO_4 , 107.4 μM EDTA-Fe, 11.5 μM MnSO_4 , 92.6 μM H_3BO_3 , 0.86 μM ZnSO_4 , 0.41 μM CuSO_4 , and 0.06 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$) adjusted to pH 6.0–6.5 was supplied every 2 days.

After confirming tuber formation (72 days later), the plant box, including the belowground part, was packed into a film bag (3 L) for gas sampling (Smart Bag PA, GL Sciences, Japan). The open portion of the bag through which the plant box was introduced was sealed with thermal crimping, except that around the stem. The remaining space between the stem and

the bag was sealed with a resin (ethylene–vinyl acetate) using a glue gun. The excess volume of the bag was minimized using a clip (GL Sciences, Japan); the gas in the bag was vacuumed out, and the bag was immediately filled with a gas mixture consisting of 240 mL of 98 atom % $^{15}\text{N}_2$ gas (Shoko Science, Japan), 60 mL of O_2 , and 1 mL of CO_2 . To prevent gas leakage, the bag including the belowground part of the plant was soaked in tap water in a container; however, the aboveground shoot was incubated in the growth chamber for an additional 10 days.

After 10 days of incubation, the belowground parts were sampled and thoroughly washed under tap water. The samples were immediately frozen in liquid nitrogen and stored at –80 °C. Approximately 0.5 g of the frozen sample of either the root or the tuber was cut and homogenized with 10 mL of distilled water using a mortar and pestle. The homogenate was passed through a filter paper (No. 6, diameter; 90 mm, ADVANTEC Inc., Japan). The filtrate (1 mL) was transferred to a 1.5-mL tube and then centrifuged at 1062 $\times g$ for 3 min. After the centrifugation, the supernatant was used for further analysis.

An aliquot of the supernatant was loaded on a stainless-steel plate and air-dried at room temperature. Raman spectra and Raman mapping images were obtained using a LabRAM HR Evolution (HORIBA, Japan) confocal micro-Raman system equipped with a 300 g/mm grating and a 100 $\times$ objective. Excitation was provided by a 532 nm Nd:YAG laser with an acquisition time of 5 s. Raman mapping was employed to generate Raman images, in which the step size was set at 1.5 μm .

Statistical analysis

Pot experiments were organized with a randomized block design, and the data were expressed as the mean $\pm$ standard error (SE) of four biological replicates. For the acetylene reduction assay, means were separated using the Tukey–Kramer test at a 0.05 probability level in JMP pro 14 (SAS Institute Inc., USA).

Results and discussion

Acetylene reduction assay and direct detection of endophytes utilizing $^{15}\text{N}_2$ with Raman spectroscopy

Nitrogenase activities were investigated using acetylene reduction assay. The roots showed approximately 5-fold higher nitrogenase activity than the other organs (Fig. 1a). However, the root biomass was very small, and the tuber

biomass represented approximately 80% of the total plant biomass (Fig. 2a–d), suggesting that the tuber may be the major site of N_2 fixation as previously suggested (Yonebayashi et al. 2014; Itoh et al. 2019).

To confirm the presence of endophytes actually fixing N_2 *in planta*, a young shoot cut from field-grown sweet potato was grown in vermiculite for 79 days in a growth chamber, and the roots and the tubers were exposed to $^{15}N_2$ (98 atom %) for 10 days. After feeding with $^{15}N_2$, resonance Raman spectroscopy detected signals of cytochrome *c* not only at 1125 cm^{-1} ($C-^{14}N$) but also at 1114 cm^{-1} ($C-^{15}N$) in the tuber extracts (Fig. 1b), as demonstrated by Cui et al. (2018). The mapping images of the tuber extract revealed that some bacteria (P 1 in Fig. 1c–f) contained $C-^{15}N$ (Fig. 1d) in addition to $C-^{14}N$ (Fig. 1e), whereas other bacteria (P 2 in Fig. 1c–f) contained mostly $C-^{14}N$ (Fig. 1e) without $C-^{15}N$ (Fig. 1d). $C-^{15}N$ signals in the bacterial cells were also detected in the root extract (Fig. 1g–j). To the best of our knowledge, this is the first direct evidence of *in planta* N_2 utilization by certain endophytic bacteria in sweet potato.

Using secondary ion mass spectrometry (SIMS), Hara et al. (2022) demonstrated that methane-oxidizing bacteria fix $^{15}N_2$ in rice roots. In combination with stable isotopes, both Raman spectroscopy and SIMS can detect and visualize metabolically active bacterial cells without dependency on microbial culture. However, unlike mass spectroscopy, Raman spectroscopy is non-destructive, which enables the investigation of active cells in the case of several single-cell-sorting technologies that are being developed (Wang et al. 2020).

Responses of biomass and N accumulation to N, P, and K inputs

The biomass of sweet potato markedly responded to the inputs of fertilizer-N and -P (Fig. 2a, b), although the response to fertilizer-K was not significant in any organ (Fig. 2c) as the K inputs did not affect the K content (Supplementary Fig. S1d). The initial soil without fertilizer-K contained a 105 mg K pot^{-1} of exchangeable K with 1 M ammonium acetate, which accounts for only 7% of total K in the whole plant grown in soil without an external K input (Supplementary Fig. S1d). Thus, sweet potato likely meets its K demand from certain soil sources other than the exchangeable fraction even in the absence of the fertilizer, resulting in a poor response to external K input and the corresponding biomass accumulation. Consequently, a greater tuber yield (81 g pot^{-1}) was obtained in the absence of fertilizer-K than without fertilizer-N (26 g pot^{-1}) or fertilizer-P (52 g pot^{-1}).

When the inputs of P and K were held constant, the tuber biomass showed a linear increase with increasing inputs of fertilizer-N up to 0.49 g N pot^{-1} (Fig. 2a). A further increase in fertilizer-N

input did not affect the tuber yield, whereas the shoot (leaf and stem) biomass showed a continuous increase (Fig. 2a). In the soil, wheat, potato, and guinea grass require fertilizer-N at approximately 0.25 g N L^{-1} soil for maximum growth (Yi et al. 2020; Igarashi et al. 2021). Based on the volume of pots (6 L) used in this study, a relatively small amount (about 1/3) of fertilizer-N is likely required for sufficient tuber production.

In contrast to the saturated responses to fertilizer-N, high inputs of fertilizer-P continuously increased the tuber as well as the shoot biomass without a saturation point, while N (0.49 g N pot^{-1}) and K inputs were held constant (Fig. 2b). We therefore reexamined the growth response in the next season with a wider range of fertilizer-P inputs under constant fertilizer-N (0.98 g N pot^{-1}) but without K fertilizer. We found a saturated response of the tuber biomass at approximately 4.0 g P pot^{-1} (Fig. 2d), where maximum yield was achieved (108 g pot^{-1}). In *Solanum tuberosum*, approximately 0.7 g P L^{-1} soil was required to attain maximum growth (Yi et al. 2019), which was almost consistent with the present study (4.0 g P in 6 L pot). These results indicate that sweet potato requires a substantial amount of available P in addition to a relatively small amount of available N for tuber production, but not K, which contradicts with the results reported by Nicholaides et al. (1985).

Water spinach (*Ipomoea aquatica*) was used as a reference plant without N_2 fixation owing to its negligible acetylene reduction activity (Fig. 1a). Six seedlings of water spinach per pot resulted in a greater biomass in shoot and root than that in sweet potato at each N input (Supplementary Table S2). Except for N content in the tuber, the N content of shoot and root was similar between water spinach and sweet potato at each N input; however, the N concentration was always higher in sweet potato (Supplementary Table S2). Although sweet potato always had a greater N content than water spinach across all treatments (Fig. 2e–g), the difference was mainly contributed by the tuber (Supplementary Table S2).

Although an N input $> 0.49\text{ g N pot}^{-1}$ did not show any further increase in N content (Fig. 2e) or tuber biomass (Fig. 2a), increasing the P input led to a linear increase in the N content (Fig. 2h). This increase in N observed despite the saturated tuber biomass (Fig. 2d), suggests that the accumulation of N is not necessarily coupled to tuber growth. The N accumulated by sweet potato reached a maximum of 1.37 g N pot^{-1} (Fig. 2h), which considerably exceeded the input of fertilizer-N (0.98 g N pot^{-1}) by about 1.4-fold. This result provides strong evidence that sweet potato acquires substantial amounts of N from sources other than soil and fertilizer.

Estimating N_2 fixation capabilities on the basis of $\delta^{15}N$ values

We determined the $\delta^{15}N$ values of whole plants to eliminate possible isotopic discriminations within a plant (Yoneyama et al. 1991; Boddey et al. 2001). Water spinach had a higher

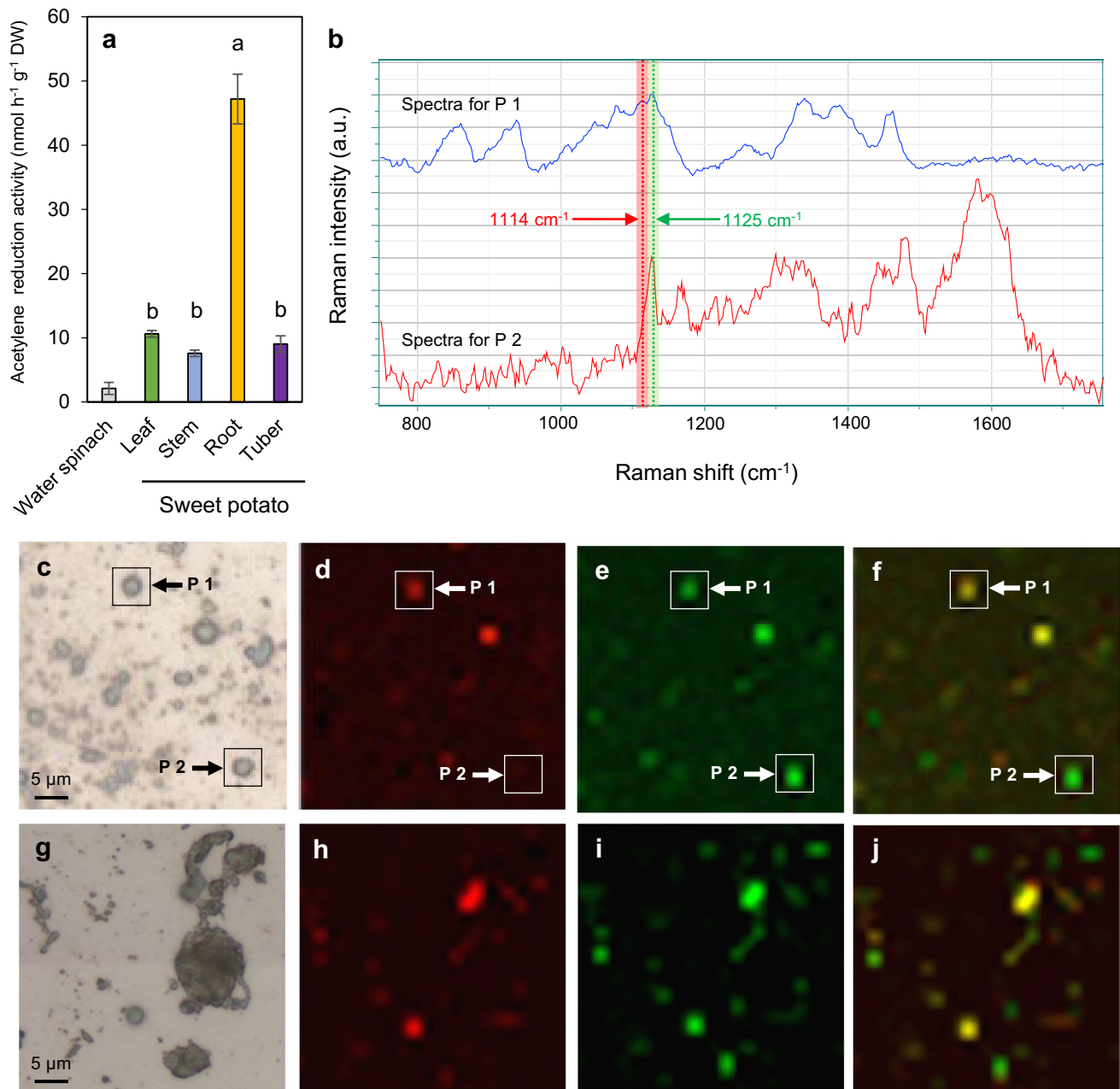


Fig. 1 Nitrogenase activity in each organ and resonance Raman spectroscopy for ¹⁵N₂-fixed endophytes in sweet potato. **a** Acetylene reduction assay (mean ± SE; *n* = 19–20 for sweet potato, *n* = 3 for water spinach) in leaves (green), stems (blue), roots (yellow), and tubers (purple) of field-grown sweet potato in comparison with pot-grown water spinach (gray). Mean values followed by the same letters are not significantly different from each other (Tukey–Kramer test; *P* < 0.05). **b** Resonance Raman spectra of single cells from P 1 and 2 in **c**, in which 1114 cm⁻¹ (C⁻¹⁵N) and 1125 cm⁻¹ (C⁻¹⁴N) signals were used to construct Raman images in **d–f** and **h–j**. **c–f** Endophytic

bacteria in the extract from the tubers of sweet potato grown in vermiculite from a young shoot cut in a growth chamber for 79 days and then exposed to ¹⁵N₂ (98 atom %) for 10 days; blight field for mapping area (**c**), corresponding Raman images mapped with C⁻¹⁵N (**d**), and C⁻¹⁴N (**e**) signals in cytochrome *c*, and their merged image (**f**). **g–j** Endophytic bacteria in the extract from the roots of sweet potato as mentioned above; blight field for mapping area (**g**), corresponding Raman images mapped with C⁻¹⁵N (**h**) and C⁻¹⁴N (**i**) signals in cytochrome *c*, and their merged image (**j**)

δ¹⁵N value (3.06 ± 0.64‰) when soil-N was its sole N source (Fig. 3a), reflecting the δ¹⁵N value of the soil-N. In contrast, the plant showed a lower δ¹⁵N value (− 1.05 ± 0.39‰) when grown in vermiculite only with the fertilizer-N as the sole

N source. Thus, the decline in δ¹⁵N values of water spinach with fertilizer-N inputs (Fig. 3a) could result from increased N derived from the fertilizer (Ndff). Using each of the mean values of δ¹⁵N as 0 and 100% of Ndff, we calculated the % Ndff

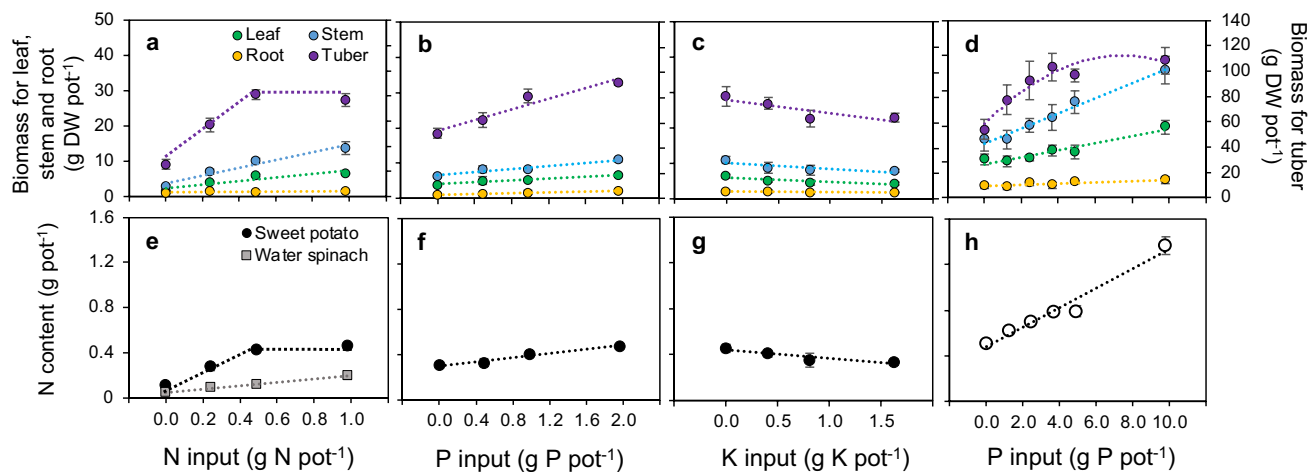


Fig. 2 Accumulation of biomass and N in sweet potato plants grown outdoors in pots. **a–d** Biomass in leaves (green), stems (blue), roots (yellow), and tubers (purple) under various inputs of N, P, and K fertilizers while holding other nutrient levels constant at 0.49 g N, 0.98 g P, or 0.81 g K per pot in 2019 (**a–c**) and at 0.98 g N per pot without K

fertilizer in 2020 (**d**). The growth period was 109 days in 2019 (**a–c**) and 99 days in 2020 (**d**). **e–h** N content of sweet potato together with water spinach in 2019 (**e–g**) and in 2020 (**h**). Each plot represents mean ± SE ($n = 4$)

of water spinach with each of the N inputs. Consequently, we obtained an Ndff of 16% at 0.24 g fertilizer-N pot⁻¹, 36% at 0.49 g fertilizer-N pot⁻¹, and 43% at 0.98 g fertilizer-N pot⁻¹, in agreement with the normal values < 50% Ndff (Yamaguchi 1991; Stevenson and Cole 1999; Yan et al. 2020).

However, the same calculations of the Ndff for sweet potato yielded 51, 74, and 86% at 0.24 g, 0.49 g, and 0.98 g fertilizer-N pot⁻¹, respectively, which are unusually high compared to the normal < 50% Ndff (Yamaguchi 1991; Stevenson and Cole 1999; Yan et al. 2020). Moreover, the Ndff calculated was 11% even in the absence of fertilizer-N. Evidently, such contradictions are caused by lower $\delta^{15}\text{N}$ values in sweet potato than in water spinach (Fig. 3a). These results also indicate that the N accumulated in sweet potato was partly derived from the atmosphere because N_2 -fixing soybean plants in the unfertilized soil had further lower $\delta^{15}\text{N}$ values ($-2.04 \pm 0.15\text{‰}$), which is consistent with the results of soybean shoots using atmospheric N_2 as the sole N source (Balboa and Ciampitti 2020).

We therefore attributed the lower $\delta^{15}\text{N}$ value in sweet potato than that in water spinach at each fertilizer-N input level to the accumulation of N derived from the atmosphere (Ndfa). Based on that, % Ndfa of sweet potato could be estimated by referring to the $\delta^{15}\text{N}$ value (-2.04‰) of the soybean plants when the % Ndfa of the soybean was known. Using a 60 to 100% Ndfa range of soybean, we simulated the N balances in sweet potato assuming a soil-N acquisition similar to that of water spinach. After fixing the amount of N derived from either the atmosphere or the soil, the amount of Ndff was calculated (Supplementary Table S3). Consequently, to meet the normal values of Ndff (< 50%) (Yamaguchi 1991; Stevenson and Cole 1999; Yan et al. 2020) in sweet potato, the % Ndfa of soybean should be > 80%, which agrees with the highest

percentage (80%) of Ndfa typically found in low-fertile soils (Stevenson and Cole 1999).

Based on the above simulations, we adopted 80% Ndfa for soybean as the minimum critical value and then calculated % Ndfa for sweet potato. Consequently, the Ndfa of sweet potato was estimated to be 11–56% (Fig. 3d–f), which generally agrees with those reported previously (Yoneyama et al. 1997; Yonebayashi et al. 2014). Furthermore, the amount of fixed-N (g Ndfa) in sweet potato was calculated by multiplying the % Ndfa by each N content. The g Ndfa was negligible without any fertilizer-N input; it increased with increasing N input but reached a saturation point (Fig. 3g) as both % Ndfa (Fig. 3d) and total N content (Fig. 2e) could no longer increase. Despite the fact that the g Ndfa did not change with more N supply above 0.49 g fertilizer-N input pot⁻¹, higher inputs of fertilizer-P enabled a further increase in fixed-N (Fig. 3h) due to the continuous increase in total N content (Fig. 1h) in addition to relatively higher % Ndfa (Fig. 3e).

The g Ndfa was negligible in the absence of fertilizer-N (Fig. 3g) when compared to the absence of either fertilizer-P (Fig. 3h) or fertilizer-K (Fig. 2i), indicating that N nutrition is critical for N_2 fixation. Considering the immediate saturated response of g Ndfa toward N inputs (Fig. 3g), it appears that the role of N nutrition could be to initiate N_2 fixation. In contrast, the role of K is unclear as K nutrition could not be altered with the inputs of fertilizer-K (Supplementary Fig. S1b, d). However, P seems to directly enhance N_2 fixation even after the N demands are met owing to its linear accumulation effect (Fig. 3h). The maximum amount of g Ndfa was estimated as 0.58 g N pot⁻¹ when the highest P input was used (Fig. 3h), which corresponds to 13 g N m⁻² being comparable to that in legumes (Yoneyama et al. 1991; Yano et al. 1994;

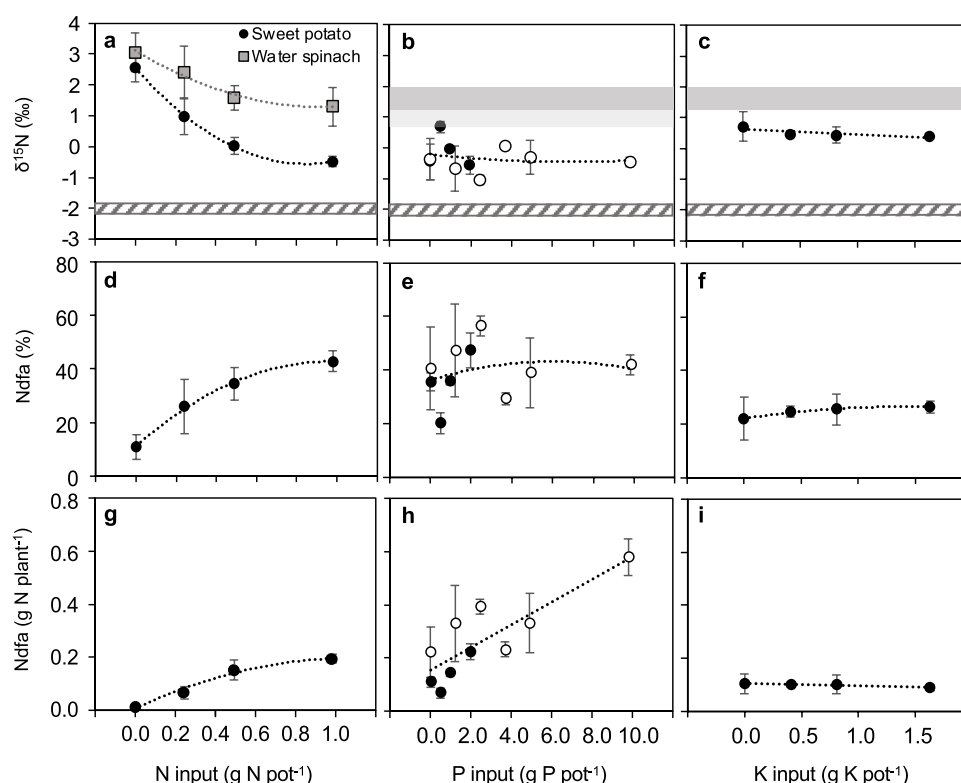


Fig. 3 Estimated N_2 -fixing capability in sweet potato based on $\delta^{15}N$. **a–c** $\delta^{15}N$ values of sweet potato in 2019 (closed) and 2020 (open), and water spinach (gray) grown outdoors in pots under various inputs of N, P, and K fertilizers while holding other nutrient levels constant at 0.49 g N, 0.98 g P, or 0.81 g K per pot in 2019, at 0.98 g N per pot without K fertilizer in 2020; the dark and light gray bands correspond to the $\delta^{15}N$ value of water spinach grown at 0.49 and 0.98 g

N per pot (**b, c**), and the cross-hatched band corresponds to the $\delta^{15}N$ value ($-2.04 \pm 0.15\text{‰}$ as the mean $\pm$ SE, $n=4$) of soybean grown in the unfertilized soil (**a–c**), respectively. **d–f** Percentages of N derived from the atmosphere among total plant N (% Ndfa) calculated from differences in $\delta^{15}N$ values among sweet potato, water spinach, and soybean. **g–i** Amounts of Ndfa in sweet potato estimated by multiplying the N content by % Ndfa. Each plot represents mean $\pm$ SE ($n=4$)

Stevenson and Cole 1999). Meanwhile, soybean used as the N_2 -fixing reference (four plants per pot) in the unfertilized soil accumulated $1.64 \text{ g N pot}^{-1}$, of which $1.32 \text{ g N pot}^{-1}$ was regarded as fixed-N, assuming 80% Ndfa for soybean.

Notably, N_2 -fixing capabilities of sweet potato were not suppressed by high inputs of N, which reduces nodule formation in leguminous plants (Marschner 1995; Daimon and Yoshioka 2008). Such insensitivity to N in sweet potato could be attributed to endophytic N_2 fixation that does not require nodule formation, even as soil N content affects bacterial diversity in the rhizosphere of sweet potato (Tangapo et al. 2018). Presently, the reason behind the strong response of endophytic N_2 fixation to P supply in sweet potato is unknown. Nitrogenase requires 16 mol ATP to fix 1 mol N_2 ; root nodules of legumes require higher amounts of P than other plant organs (Marschner 1995). Although sweet potato has no specific organ to fix N_2 , a remarkable increase in P concentration with elevated P supply was observed in the stem (3.3-fold) and the tuber (2.6-fold) than in the root (2.0-fold) and the leaf (1.7-fold) tissues (Supplementary Fig. S1a, c). Endophytes are assumed to colonize the intercellular spaces of the apoplast (McCully 2001). Therefore,

it would be worthwhile to investigate the cellular level P concentrations in the vicinity where endophytes actually fix N_2 .

Conclusions

Here, we have demonstrated that N_2 fixation in sweet potato is P-dependent after meeting a smaller N demand than that of cereals, exhibiting a maximum capability comparable to that of legumes. The results are important because they suggest that high N_2 -fixation as exhibited by legumes is possible even in the absence of specialized organs such as root nodules. The tuber seems to be a major site of N_2 fixation considering an increased accumulation of biomass together with the acetylene reduction activity, although the activity was rather higher in the roots. Thus, there appears to be a positive correlation between N_2 fixation and tuber growth. However, a linear increase in N content is possible regardless of the saturated response of the tuber biomass to P input, suggesting that N_2 fixation and tuber growth could be decoupled in sweet potato. Using resonance Raman spectroscopy,

we have provided direct evidence of $^{15}\text{N}_2$ fixation by certain endophytes in sweet potato for the first time. The method enables non-destructive visualization of bacterial cells that actively fixed N_2 *in planta*. Future studies should focus on identifying the bacterial endophytes as well as understanding their diversity and community structure.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00374-023-01698-5>.

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Author contribution K.Y. and R.U. conceived the study. R.U. performed the experiments and contributed to data analysis and manuscript writing. K.Y. supervised the experiments, contributed to funding acquisition, experimental design, and data analysis, and wrote the manuscript.

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Data availability The data supporting this study are available in the “Supplementary Information.”

Declarations

Conflict of interest The authors declare no competing interests.

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