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SHORT NOTE

Mycorrhizal status of Cyperaceae from New Caledonian ultramafic soils: effects of phosphorus availability on arbuscular mycorrhizal colonization of *Costularia comosa* under field conditions

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Abstract Plants from the Cyperaceae family (sedges), usually considered as non-mycorrhizal, constitute almost exclusively the herbaceous stratum of the ultramafic maquis in New Caledonia. These plants are pioneers and are important for the ecological restoration of mined areas. *Costularia comosa*, one of the most common sedges in this environment, was grown under field conditions on ultramafic soil, fertilized or not with phosphate and/or nitrogen. Results showed that the addition of phosphate to the soil induced a clear increase in mycorrhizal colonization of *C. comosa* and an increase in arbuscule abundance, reflecting the establishment of a functional mycorrhizal symbiosis. Significant positive correlations were found among mycorrhizal parameters and plant or soil phosphorus concentrations. Nitrogen fertilization did not affect mycorrhizal colonization of *C. comosa*. The improvement in mycorrhizal colonization by phosphate fertilization did not influence significantly nickel concentrations in the roots and shoots of plants. This study demonstrated that phosphate fertilization of ultramafic soil improved mycorrhizal colonization of *C. comosa*, with formation of a functional symbiosis under field conditions.

Keywords Ultramafic soils · Arbuscular mycorrhiza · Phosphorus · Cyperaceae · *Costularia* · Field experiment

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Introduction

Ultramafic ecosystems (serpentine) occupy one third of the main island in the tropical South Pacific island group of New Caledonia. Ultramafic soils are characterized by very low concentrations of phosphate (P), nitrogen (N), and Ca and high levels of Ni, Co, Mn, and Cr (Brooks 1987). About 89 % of the vascular plants in the ultramafic maquis are endemic to New Caledonia (Jaffré et al. 2004). Among them, Cyperaceae species (sedges) are pioneers and constitute almost exclusively the herbaceous stratum (Jaffré 1992). These plants are therefore potentially important for the revegetation of mined areas. *Costularia* is the most widespread genus of the Cyperaceae family in ultramafic maquis in New Caledonia, with 12 species being endemic (Jaffré et al. 2004). *Costularia comosa* (C.B. Clarke) Kük is frequently found in all the ultramafic regions of New Caledonia and is particularly recommended for ecological restoration (Wulf et al. 2010).

Cyperaceae are generally considered as non-mycorrhizal or very weakly mycorrhizal (Miller et al. 1999; Wang and Qiu 2006; Brundrett 2009). However, some publications have reported the presence of arbuscular mycorrhizal (AM) associations within Cyperaceae (Amir et al. 1997; Muthukumar et al. 2004; Gai et al. 2006; Perrier et al. 2006). Lagrange et al. (2011) demonstrated that even a low level of mycorrhizal colonization is functional in *C. comosa* plants grown in ultramafic soil under greenhouse conditions. These authors hypothesized that in natural conditions, mycorrhizal colonization of Cyperaceae could be inhibited by phosphorus deficiency, as suggested by Lambers et al. (2009) for extremely poor soils.

Previously reported field experiments on ultramafic soils have concerned the effects of fertilization on growth and plant species dynamics (Ferreira and Wormell 1971; Carter

et al. 1988; Chiarucci and Maccherini 2007), without considering mycorrhizal symbiosis. The aim of the present study was to evaluate the effects of mineral phosphate and/or nitrogen fertilization on the mycorrhizal status of *C. comosa*, under field conditions on ultramafic soils.

Materials and methods

Experimental site description

New Caledonia is located approximately 1,500 km east of Australia and 1,800 km north of New Zealand in the South Pacific. The climate is subtropical to tropical, with a rainy season from December to May and a dry season from September to November that can vary in duration and severity. Mean monthly temperature in the capital, Noumea, is highest in February with 26.2 °C and lowest in August with 19.9 °C. The experimental site is located in an ultramafic ligno-herbaceous maquis in the plum area (22°16′59″S, 166°39′12″E). The soil of this site is a highly weathered geric ferralsol (Becquer et al. 2001) with the following physicochemical characteristics: 4.56 % organic carbon; 0.22 % total N; C/N 20.0; pH 5.34 (H_2O); 192.4 mg kg^{-1} total P; 3 mg kg^{-1} available P (Olsen); 0.09 % Ca; 0.72 % Mg; 93.6 mg kg^{-1} Na; 682.7 mg kg^{-1} K; 37.8 % Fe; 1.43 % Mn; 0.54 % Ni; 0.12 % Co; 3.67 % Cr; 170 mg kg^{-1} Mg (dimethylenetriaminopentaacetic acid (DTPA)); 1,116 mg kg^{-1} Mn (DTPA); 106 mg kg^{-1} Fe (DTPA); 116 mg kg^{-1} Ni (DTPA); 79 mg kg^{-1} Co (DTPA); and 0.6 mg kg^{-1} Cr (DTPA).

Field experiment

The experiment aimed at assessing the effect of P and/or N fertilization on the mycorrhizal status and growth of *C. comosa* in ultramafic soil conditions formed part of a project of field production of Cyperaceae seeds for ecological restoration conducted by the firm SIRAS Pacific and the experimental plan involved other aspects not presented here (plant flowering, seed production, etc.). The natural vegetation was removed and the soil homogenized using agricultural equipment; any invading plants were regularly removed. A completely randomized experimental block design typically used for agronomic assays was chosen to take into account the heterogeneity of the experimental field (Dagnélie 2003). The experimental field was divided into five blocks: one block represented a homogeneous and flat planting area bounded by 2.5 m wide strips of natural vegetation (maquis). These strips were left in their natural state to minimize erosion and runoff, which could induce transfer of mineral inputs and particles from one block to another. The field sloped gently (5 %); the five flat blocks being perpendicular to the slope. Each block was divided into four plots separated by a 1-m-wide path and corresponding to the four combinations of N and P fertilization treatments, arranged randomly. Each plot was an experimental unit of 12 m^2 containing 24 plants of *C. comosa*, corresponding to one treatment. The plants were distributed in two planting lines, 50 cm apart. Overall, for each treatment, 5 plots of 24 plants were assessed.

C. comosa plants were produced in a nursery and were aged 12 months when planted into the field in April 2006. *C. comosa* seeds were obtained from an ultramafic maquis near lake Yaté (south New Caledonia). Plants were firstly grown 16 months in a nursery (the plants grow very slowly) at 23–28 °C, in small pots (170 cm^3) containing a mix of coco fiber (30 % *v*/*v*), commercial compost (10 %), and old topsoil (60 %), before planting them in the experimental field. The level of mycorrhizal colonization of the plants before transplanting was estimated to be less than 0.5 %.

The four fertilization treatments were: N−P− (control without fertilization), N−P+, N+P−, and N+P+. The nitrogen fertilizer used was "medium Azolon 38 N," a urea–formaldehyde-based slow-release fertilizer (12 months under the influence of temperature and humidity) containing 38 % of total N (2 % of N as urea and 36 % as methylene urea). Nitrogen (N+ treatment) was added at 500 kg of N per ha. Phosphorus (P+ treatment) was added as triple superphosphate (N=0 %, P_2O_5=48 %, K_20=0 %) corresponding to 218 kg P per ha (500 kg P_2O_5). This supply was high in comparison with levels generally used because P is very strongly adsorbed by iron oxides in ultramafic soils, especially in New Caledonia, where levels of available P are very low (Dubus and Becquer 2001; Jaffré and L'Huillier 2010a, 2010b). Potassium was added as sulfate of potash (K_2O=50 %) at 200 kg K_2O per ha for all treatments, so that the potassium deficiency could not act as a limiting factor. An irrigation system consisting of a network of drip lines controlled by solenoid valves was established to standardize watering and support the development of plants. This automated system delivered 0.5 L of water per day to each plant, except on rainy days.

Plant sampling and chemical analysis

Two years after planting, roots and leaves of three plants per plot were sampled (15 plants per treatment). Whole plants were taken randomly from the experimental layout, roots were washed several times separately from leaves to remove soil particles, and plants were dried at 60 °C for 3 days. The dry weight of both roots and leaves was measured, and then, each plant part was ground to determine total N (Kjeldahl), total P, and total Ni by inductively coupled plasma optical emission spectroscopy (ICP-OES; Optima 3300 DV, PerkinElmer) at the chemistry laboratory of Institut de Recherche pour le Développement (IRD) (Laboratoire des Moyens Analytiques (LAMA) IRD, Noumea).

Root sampling and assessment of mycorrhizal colonization

For each treatment, fine roots of the 15 sampled plants were taken to assess mycorrhizal colonization following staining with Trypan blue (Koske and Gemma 1989 modified). Root fragments were placed in 10 % KOH at 90 °C for 90 min; a few drops of hydrogen peroxide (H_20_2) were added after 1 h to clear the brown color. The roots were then rinsed in distilled water and stained with Trypan blue (15 min at 90 °C). For each plant, at least 25 root segments of 1–2 cm length were mounted in 10 % glycerol on glass slides and observed under an optical microscope. A root segment was considered to be mycorrhizal when arbuscules, vesicles, or intracellular hyphal coils were clearly identified. The intensity of arbuscular mycorrhizal colonization, i.e., mycorrhizal intensity (M %), was determined according to Trouvelot et al. (1986). $M\ (\%) = \frac{95n5+70n4+30n3+5n2+n1}{\text{total number of root fragments}}$ where n5 is the number of root fragments with more than 90 % root colonization, n4 between 90 and 50 % root colonization, n3 between 50 and 10 % root colonization, n2 between 10 and 1 % root colonization, and n1 less than 1 % root colonization. Arbuscule abundance (A %) was also estimated using the same formula, but considering only the presence of arbuscules instead of global mycorrhizal colonization.

Soil sampling and chemical analysis

A volume of 1,500 cm^3 of soil was collected near the plants, at 0–30 cm depth, for soil analyses. Three samples were collected at different locations in each plot and mixed to obtain a composite sample representative of the plot. The samples were air dried immediately after collection and sieved through a 2-mm mesh before analysis at the chemistry laboratory of IRD (LAMA IRD, Noumea). Total and available P (Olsen) were measured by ICP-OES 3 months, 1 year, and 2 years after planting.

Statistical analysis

Data, expressed as mean and standard deviation of the mean, were compared using a variance analysis with the statistical software V2.0 beta Kiplot 13. Data were checked for normal distribution. One-way ANOVA including the Tukey test was carried out for each parameter separately and a total correlation test to analyze the correlations between the parameters studied.

Results

P and N fertilization clearly improved growth of *C. comosa* and plant biomass in phosphate- and nitrogen-fertilized plots was more than three times greater than in the non-fertilized control plot (Table 1). However, leaf dry weight did not differ between the fertilization treatments due to the high variability of the values. P concentration in roots was significantly higher in P-treated plots than in non-fertilized plots. The P concentrations in leaves were similar in all the treatments, but the amount of P absorbed per plant (P content) was nearly three times higher in plots fertilized with P and N. N concentrations in roots and leaves did not vary according to the fertilization treatment, but plants absorbed generally more N in plots in which N and P were added. No differences were observed for Ni concentrations in roots and leaves between the four treatments.

The average total P in soil at the beginning of the experiment was 154 mg kg^{-1}, but only 1.8 mg kg^{-1} was available. After 1 year, these values decreased or were maintained at the same level in non-fertilized plots (127 mg kg^{-1} of total P; 3.7 mg kg^{-1} of available P) whilst the P-fertilized plots (N+P+, N−P+) showed significantly higher levels, in particular for available P with concentrations four to seven times higher than in control non-fertilized plots (Table 1). There were no significant differences between P- and N-fertilized plots (N+P+) and those that only received P (N−P+). After 2 years, the values of total and available P were not markedly different from those obtained after 1 year.

The values for mycorrhizal intensity were very low for plants in the control plots (2.9 %), but the addition of P greatly increased these values to 18.2 and 21.2 % for the N−P+ and N+P+ treatments, respectively (Table 1). P fertilization also stimulated arbuscule production in mycorrhizal roots (values about four times higher than the control). Correlations between mycorrhizal and plant nutrition parameters are presented in Table 2. There were no significant correlations between *C. comosa* plant growth and mycorrhizal colonization. Multiple regression analysis was also performed and did not reveal any significant additive effects (multiple correlations), particularly between growth parameters, phosphate fertilization, and mycorrhizal colonization. P concentrations in leaves and in roots were highly positively correlated to mycorrhizal intensity and arbuscule abundance ($P<0.05$) indicating that plants with the highest level of mycorrhizal colonization absorbed more P. Mycorrhizal intensity and arbuscule abundance were also generally positively correlated to total and available P in soil ($P<0.05$), at the beginning of the experiment and after 1 or 2 years (Table 2).

N concentration in roots (but not N concentration in leaves) was also positively correlated to mycorrhizal parameters. No correlations were observed between mycorrhizal colonization and Ni concentration in leaves and roots.

Discussion

Many plant species endemic to New Caledonia's ultramafic maquis are characterized by low growth rates. This is

Table 1 Growth, nutrition, and mycorrhizal colonization characteristics of *C. comosa* plants grown in field, with or without mineral fertilization

	N−P−	N−P+	N+P−	N+P+
Total dry weight (g plant^{-1})	27.9±8.9 a	48.1±31.9 ab	45.3±31.7 ab	96.5±49.0 b
Leaves dry weight (g plant^{-1})	15.4±5.7 a	27.6±18.3 a	31.1±21.1 a	37.83±25.5 a
P in leaves				
mg kg^{-1}	220±28 a	288±40 a	199±40 a	269±77 a
mg plant^{-1}	3.4±1.2 a	7.5±4.4 ab	6.3±5.5 ab	9.8±6.9 b
P in roots				
mg kg^{-1}	136±10 a	207±51 b	120±15 a	237±64 b
mg plant^{-1}	0.9±0.5 a	2.3±0.9 ab	0.9 ±0.6 a	6.6±2.5 b
N in leaves				
g kg^{-1}	8.9±0.3 a	8.2±0.9 a	8.2±1.1 a	7.9±1.2 a
mg plant^{-1}	138.1±50.3 a	215.2±134.9 a	258.5±196.8 a	293.1±191.4 a
N in roots				
g kg^{-1}	3.3±0.3 a	3.8±0.9 a	3.8±0.4 a	3.8±1.7 a
mg plant^{-1}	21.9±11.9 a	44.4±21.5 ab	26.9±15.1 a	107.2±54.4 b
Ni in leaves (mg kg^{-1})	25.8±8.1 a	28.6±6.3 a	26.0±3.1 a	23.0±5.2 a
Ni in roots (mg kg^{-1})	196.2±48.1 a	231.2±99.4 a	206.6±42.4 a	246.0±66.0 a
Total P in soil after 3 months (mg kg^{-1})[a]	243.2±123.7 a	230.9±85.0 a	189.4±107.3 a	177.4±161.3 a
Available P in soil after 3 months (mg kg^{-1})[a]	19.2±19.0 a	13.6±12.2 a	10.2±9.0 a	13.4±12.1 a
Total P in soil after 1 year (mg kg^{-1})	127.8±10.2 a	231.0±40.9 b	114.1±20.8 a	291.1±83.8 b
Available P in soil after 1 year (mg kg^{-1})	3.7±1.3 a	15.6±5.9 b	4.2±1.3 a	27.5±14.8 b
Total P in soil after 2 years (mg kg^{-1})	167.2±22.3 a	313.4±114.5 b	170.0±25.2 a	249.8±107.3 ab
Available P in soil after 2 years (mg kg^{-1})	3.7±1.3 a	22.9±18.9 b	3.2±1.3 a	16.9±13.6 b
Mycorrhizal intensity (M %) after 2 years	2.9±2.7 a	18.2±15.5 b	5.4±5.4 a	21.2±9.5 b
Arbuscule abundance (A %) after 2 years	1.0±1.0 a	4.7±4.2 b	1.9±1.5 ab	3.9±1.5 b

[a] Just before the addition of NPK and before planting, total P in soil was 154.7 mg kg^{-1} and available P in soil was 1.8 mg kg^{-1}. For each parameter, means with different letters are significantly different (Tukey–Kramer test, $P<0.05$)

Table 2 Total correlations (Pearson correlation coefficient) between mycorrhizal parameters in the one hand and plant growth, plant nutrition parameters, and P contents in soil in the other (all treatments taken together, 60 values)

		Mycorrhizal intensity, M (%)	Arbuscule abundance, A (%)
Plant parameters	M (%)	1	0.833
	Total dry weight	−0.149	−0.82
	Leaves dry weight	−0.231	−0.146
	P concentration in leaves	**0.399****	**0.348****
	P concentration in roots	**0.634*****	**0.450*****
	N concentration in leaves	0.060	0.084
	N concentration in roots	**0.553*****	**0.332****
	Ni concentration in leaves	−0.169	−0.022
	Ni concentration in roots	−0.40	0.154
Soil parameters	Total P in soil after 3 months	**0.336****	0.240
	Total P in soil after 1 year	**0.370****	**0.313***
	Total P in soil after 2 years	**0.331****	**0.404****
	Available P in soil after 3 months	**0.324***	0.132
	Available P in soil after 1 year	**0.301***	**0.299***
	Available P in soil after 2 years	0.169	**0.270***

Multiple regression analysis was also done and did not reveal significant additive effects. Significant correlations are in bold
*$P<0.05$, **$P<0.01$, ***$P<0.001$ ($n=60$)

especially true for *C. comosa*, which can grow with very low levels of main elements and can be considered as oligotrophic (Jaffré and L'Huillier 2010a, 2010b). In the present study, the P concentration in *C. comosa* leaves was low (0.022 %) and did not increase significantly in fertilized plots (0.028 %). Available P was strongly deficient in the ultramafic soil (only 1.8 mg kg^{-1}, before fertilization) in relation to its pH (5.3) and to the strong adsorption to iron oxides (Dubus and Becquer 2001; Jaffré and L'Huillier 2010a, 2010b). One year after fertilization, total P values in soil were about twice than that recorded for non-fertilized plots and available P was about ten times higher, but stayed relatively low (15–27 mg kg^{-1}). Under these conditions, *C. comosa* growth was stimulated by P supply, particularly when N was also added. The high variability of the values obtained for different parameters, especially for plant dry weight, can be related to soil heterogeneity as underlined by Lagrange (2009) from physicochemical analyses of different samples taken in the studied experimental field.

AM fungal propagules are relatively abundant in New Caledonian ultramafic soils (Perrier et al. 2006; Amir and Ducousso 2010). It is the case with the soil studied here which was used as natural inoculum by Lagrange et al. (2011) and induced a relatively good mycorrhizal colonization in sorghum plants (M=37 %). The low level of mycorrhizal colonization (<3 %) of *C. comosa* plants in non-fertilized plots could then be considered as an illustration of the low affinity of Cyperaceae species with AM fungi (Miller et al. 1999; Wang and Qiu 2006). However, it is important to point out that mycorrhizal intensity in the *C. comosa* plants increased to 18–21 % in P-amended plots. This stimulation of mycorrhizal colonization can be related to P supply, since it was not significantly enhanced by N when added without P. The concomitant increase in arbuscule abundance in P-amended plots indicates that the AM fungi form a functional symbiosis (Brundrett 2009). Indeed, P concentrations in roots and leaves were highly positively correlated with mycorrhizal intensity and arbuscule abundance so that plants having the highest overall mycorrhizal colonization absorbed more P, suggesting a role of the fungal symbionts in P uptake, as for many AM plant species (Bolan 1991; Ezawa et al. 2002; Smith and Read 2008). The absence of a non-mycorrhizal control treatment for each of the four fertilization treatments, in the context of a field experiment, does not allow a direct evaluation of the effects of AM fungal colonization on P uptake by *C. comosa*. Root/shoot ratios are frequently reduced by AM fungal colonization, in comparison with non-mycorrhizal plants (Smith and Read 2008; Veresoglou et al. 2012). This was not the case when AM fungal colonization increased in *C. comosa* in the P-fertilized plots (root/shoot ratios 0.811 for N−P− and 0.742 for N−P+). On the contrary, in N- and P-fertilized plots (N+P+), the root/shoot ratio clearly increased (1.551). However, under these conditions, it is difficult to distinguish the direct influence of P supply on the plants from the effect of the higher AM fungal colonization. Indeed, increased P supply to plants can induce root proliferation (Drew 1975; Sun et al. 2002; He et al. 2003). Nevertheless, P uptake efficiency by *C. comosa* plants (total P absorbed per milligram roots) was improved in plots only fertilized with P (0.344 for N−P−; 0.478 for N−P+), but not in the N- and P-fertilized plots (0.279 for N+P+) where the increase in root biomass could have masked the effect of AM fungi on P absorption. In addition, the efficiency of the mycorrhizal pathway for P uptake will depend not only on arbuscule abundance for transfer to the plant, but also on the amount of extraradical hyphae involved in uptake from the soil and translocation to roots, which was not estimated in the present study.

The significant positive correlations between mycorrhizal parameters with the total and available P in soil indicate that *C. comosa* root colonization by AM fungi is promoted in soils with the highest levels of P, suggesting that P deficiency in soil reduces the ability of this plant species to form mycorrhiza. It is well known that high levels of P can inhibit mycorrhizal colonization in other plants (Marschner 1995), but the effects of P deficiency on mycorrhizal colonization have not been clearly demonstrated. Under different conditions, Schubert and Hayman (1986) considered that mycorrhizal development in onion plants was optimal at 50 mg kg^{-1} of available P in agricultural soils, whilst other studies have reported a relatively high level of mycorrhizal colonization of other plants in highly P-deficient soils (Vivas et al. 2003; Chaudhry et al. 2005; Zangaro et al. 2008; Pagano et al. 2010). It is clear that strong P deficiency cannot always be a limiting factor for mycorrhizal colonization. However, the present results suggest that, under particular conditions like those in ultramafic soil, strong P deficiency may reduce root colonization by AM fungi, at least for plant species like *C. comosa*.

Lambers et al. (2009) suggested that mycorrhiza strategies may not be effective for the plant in highly P-deficient soils and considered that specialized roots, such as cluster or dauciform roots, in non-mycorrhizal plant species could be more efficient for P absorption in these conditions. The root system of *C. comosa* develops dauciform roots that may be colonized by AM fungi (Lagrange et al. 2011). Dauciform roots were observed in *C. comosa* in the present field experiment, without differences in their development between P-fertilized or non-fertilized plots, indicating that plants were still under P deficiency stress (Neumann and Martinoia 2002), probably because of the very strong adsorption of added P to iron oxides in this type

of soils (Dubus and Becquer 2001; Jaffré and L'Huillier 2010a, 2010b).

It is not clear whether the increase in *C. comosa* biomass in fertilized plots is only related to P and N supply or if AM fungal activity contributed to this effect. No multiple correlations were found between these parameters. The absence of a significant correlation between mycorrhizal colonization and plant growth, as noted here, is relatively frequent (Smith and Smith 2010) and can be related to the high variance inside each group of values, reflecting interactions with a large number of variables, as is frequently the case in field experiments.

In this study, nickel concentration in leaves and roots of *C. comosa* was not negatively correlated to mycorrhizal colonization, which does not support the results of Lagrange et al. (2011) who found that Ni concentration in roots of *C. comosa* grown under greenhouse conditions was reduced by inoculation with AM fungi. It is possible that the high variability in values under field conditions could mask the protective effect of AM against nickel toxicity which has been reported also for other plant species endemic to New Caledonia's ultramafic maquis (Amir and Ducousso 2010; Amir et al. 2012).

In conclusion, the present study shows that the mycorrhizal status of the sedge species *C. comosa* varies in relation to available P concentration in ultramafic soil. Brundrett (2009), discussing the mycorrhizal status of the Cyperaceae family, proposed different hypotheses to explain the variable reports concerning this status (NM-AM). One of them is that AM fungi can be found at low frequencies in Cyperaceae plant roots, but they do not form arbuscules and are not functional, behaving as endophytic or saprophytic organisms. This hypothesis is in contradiction with the present and previous observations (Lagrange et al. 2011). The second hypothesis is that Cyperaceae are potentially AM, but often occur in mycorrhiza-suppressive habitats. The present results clearly support this hypothesis, since *C. comosa* is generally weakly mycorrhizal in natural conditions (Perrier et al. 2006; Lagrange et al. 2011) with very low levels of soil P, but its mycorrhizal status is clearly AM in P-fertilized soil. The positive effect of moderate P fertilization on mycorrhizal colonization of *C. comosa* could be useful for ecological restoration of degraded mining areas. Indeed, this plant is one of the species most used for this purpose, and current experiments aim at determining the optimal doses of mineral P which may stimulate mycorrhizal colonization as well as plant growth.

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