Hindawi Publishing Corporation
International Journal of Agronomy
Volume 2014, Article ID 507405, 10 pages
http://dx.doi.org/10.1155/2014/507405

Research Article

Root Distribution and Nitrogen Fixation Activity of Tropical Forage Legume American Jointvetch (*Aeschynomene americana* L.) cv. Glenn under Waterlogging Conditions

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Received 16 April 2014; Revised 3 August 2014; Accepted 17 August 2014; Published 8 September 2014

Academic Editor: Ravindra N. Chibbar

We investigated the root distribution and nitrogen fixation activity of American jointvetch (*Aeschynomene americana* L.) cv. Glenn, under waterlogging treatment. The plants were grown in pots under three different treatments: no waterlogging (control), 30 days of waterlogging (experiment 1), and 40 days of waterlogging (experiment 2). The plants were subjected to the treatments on day 14 after germination. Root dry matter (DM) weight distribution of waterlogged plants was shallower than controls after day 20 of waterlogging. Throughout the study period, the total root DM weight in waterlogged plants was similar to that in the controls. Enhanced rooting (adventitious roots) and nodule formation at the stem base were observed in waterlogged plants after day 20 of waterlogging. The average DM weight of individual nodules on the region of the stem between the soil surface and water surface of waterlogged plants was similar to that of individual taproot nodules in the controls. Waterlogged plants had slightly greater plant DM weight than the controls after 40 days of treatment. The total nitrogenase activity (TNA) of nodules and nodule DM weight were higher in waterlogged plants than in the controls. Waterlogged American jointvetch had roots with nodules both around the soil surface and in the area between the soil surface and water surface after 20 days of waterlogging, and they maintained high nitrogenase activity and net assimilation rate that resulted in an increased growth rate.

1. Introduction

In Japan, since the 1970s, rice production has undergone many changes, and parts of paddy fields have been used for production of field crops or forage. In recent years, forage production in upland paddy fields has increased. These upland paddy fields comprise approximately 18% of the total forage production area in Japan [1]. They are characterized by poor drainage or high ground-water table that results from an inflow of water from the neighboring paddy fields. This inflow of water leads to a very wet soil. Therefore, forage species with high tolerance to wet soils are required in order to maximize the production in these fields. Forage legumes, which introduce nitrogen into pasture and cropping systems, are an integral component of animal feeds, and they make an important contribution to the nutritive value of pastures [2–5]. However, there are few studies on the cultivation and utilization of tropical forage legumes in drained paddy fields in southern areas of Japan [6].

During waterlogging, the oxygen concentration in soils decreases, and plant roots experience hypoxia. Plants with roots with low tolerance to waterlogging do not prosper well in wet soils due to decreased root respiration and mineral absorption [7–11]. Wetland plants and some upland plants can survive under flooding conditions because of the well-developed aerenchyma tissue in their roots [12–17]. A white, spongy tissue with substantial gas space covers the stem base, roots, and nodules of some flood-tolerant leguminous plants, such as *Viminaria juncea* [18], *Lotus uliginosus* [19, 20], *Lotus corniculatus*, *Lotus glaber* [21], and *Glycine max* [14, 15, 22]. Under waterlogging conditions, this spongy tissue is connected with the aerenchyma tissues at the nodes. Therefore, this tissue is regarded as a continuous aerenchymatous link and is called secondary aerenchyma

[15, 23]. Plants growing in waterlogged conditions have shallow roots that are distributed near the soil surface [6, 24–26]. Swelling of the submerged portions of the lower shoot [27] and development of hypertrophic lenticels [27, 28] facilitate oxygen entry into the aerenchyma of nearby adventitious roots [29]. The swelling of the submerged portions of the lower shoot is probably a consequence of cell expansion promoted by endogenous ethylene formed in the submerged tissue by water [28, 29]. A more widespread response is the emergence, from the shoot base, of aerenchymatous adventitious roots to replace longer and deeper roots damaged or killed due to anoxia [29, 30]. In grains such as maize, ethylene promotes the outgrowth of root primordia from the stem base [29]. The survival of some species under flooding conditions has been attributed to shallow rooting [31]. The wheat lines with shallow roots show higher tolerance to waterlogging than those with deep roots [32].

Aeschynomene americana L. (American jointvetch) cv. Glenn is an erect-ascending, annual or short-lived perennial, shrub-like tropical legume. These plants are native to Central America and the tropical areas of South America, extending from as far south as Argentina to Florida, USA, in the north, and the West Indies [33]. Essentially as a wetland plant, it requires a minimum of 1000 mm of mean annual rainfall [33]. *Aeschynomene americana* L. cv. Glenn is a self-regenerating annual legume that is released from Florida, USA [34]. This plant has a high tolerance to wet conditions [34–37] and produces a high quantity of dry matter [33, 38]. Albrecht et al. [39] reported that 20-day waterlogged *A. americana* showed similar nitrogen fixation activity as the controls. Shiferaw et al. [40] also reported that 7- and 14-day waterlogged *A. americana* cv. Glenn showed similar total plant weight and nodule dry weight as the controls. However, there is insufficient information about the growth, root distribution, and nitrogen fixation activity of American jointvetch subjected to more than 30 days of waterlogging. Understanding the growth characteristics of this legume under such conditions will contribute to the management of the productivity of cultivation and livestock in the tropical and subtropical regions of the world.

In this study, two experiments were conducted to diachronically investigate the root distribution and nitrogen fixation activity of American jointvetch (cv. Glenn) in a waterlogged soil for 30 days (experiment 1) or 40 days (experiment 2). A part of experiment 1 had already been reported [35], but it was used here to discuss the results obtained in the present study because little is known about the distribution of roots and nodules according to soil depth.

2. Materials and Methods

2.1. Experiment 1. A part of this experiment has already been reported [35], and its outline is given below. American jointvetch was sown on May 20, 1992, in pots (inside diameter 12.5 cm, height 40.0 cm, 3 plants per pot) filled with rice paddy soil (heavy soil, pH 5.6; total N, 0.13%; C/N, 13.85; total P, 0.03%), and grown in a phytotron (Biotron Institute, Kyushu University, Japan) under natural light conditions at 25°C and 70% relative humidity. Superphosphate and potassium chloride fertilizers were added to the soil in each pot (0.123 g pot^{-1} of P and K). Rhizobia were not inoculated because it was shown that adequate amounts of rhizobia strain suitable for this plant (cowpea type) exist in paddy soil [38].

There were two treatments each with three replicates. Pots in the nonwaterlogging treatment group (control) were watered daily to field capacity and allowed to drain freely, while pots with 30-day waterlogging treatment were watered daily to maintain the water level at 2 cm above the soil surface and the bottom of the pot was corked. Treatments began on day 14 after emergence.

Plants were collected at the start of the treatment (0) and 10, 20, and 30 days later. Dry matter (DM) weight (70°C, 72 h) and root distribution were measured. Plants were cut at the soil surface and the roots were cut every 5 cm from the soil surface to a soil depth of 40 cm. Root parts were washed and soil was carefully removed. Nodules were separated from roots for measurement of DM weight.

2.2. Experiment 2. American jointvetch sown on May 11, 1995, in pots (1/5000a Wagner pot, 3 plants per pot) filled with paddy soil (same as experiment 1) was grown in a phytotron (Biotron Institute) under natural light conditions at 25°C. Superphosphate and potassium chloride fertilizers were added to the soil in each pot (0.2 g pot^{-1} of P and K).

There were two treatments, each with three replicates. Pots in the nonwaterlogging treatment group (control) were watered daily to field capacity and allowed to drain freely, while pots with 40-day waterlogging treatment were watered daily to maintain the water level at 2 cm above the soil surface and the bottom of the pot was corked. Treatments began on day 14 after emergence. Like in experiment 1, rhizobia were not inoculated in experiment 2 as well.

Soil oxidation-reduction potential (Eh) was measured from the start of the treatment (0) to 40 days of waterlogging treatment by ORP electrode (9300-10D, Horiba Inc., Kyoto, Japan). Plants were collected at the start of the treatment (0) and 2, 5, 11, 20, and 40 days later. DM weight and nitrogen fixation activity were measured with the acetylene reduction method [35, 41]. Root distribution was determined by separating the lateral roots from the taproot. The total nitrogenase activity (TNA) of nodules was measured separately in the above-ground parts and subterranean parts of the plants. Each part of the plant was put into a 1-L glass jar, and the air in the jar was adjusted so that it contained 5% acetylene. Plants were then incubated for 1 h at room temperature (about 25°C). Then, 1.0 mL of gas was collected from the jar and injected into a gas chromatograph (GC-6A, Shimazu Inc., Kyoto, Japan; with Porapak Q + Porapak N, conditions: injection temperature 50°C, column temperature 50°C, and FID detection temperature 50°C) to measure the volume of ethylene. Specific nitrogenase activity (SNA) was calculated from TNA and nodule DM weight.

2.3. Statistical Analysis. Data obtained on the same day from the controls and from the plants subjected to waterlogging treatments were analyzed using the t-test (plant DM weight, basal stem diameter, root DM weight, nodule DM weight, TNA, and SNA) and one-way analysis of variance (ANOVA; individual part DM weight, root and nodule DM weight, and TNA). Significant differences were subjected to Fisher's protected LSD tests (Super ANOVA, v. 1.11; Abacus Concepts, Berkley, CA, USA).

3. Results

In experiment 2, soil oxidation-reduction potential (Eh) decreased rapidly with increasing duration of waterlogging treatment (3 d = −38 mV, 6 d = −143 mV, 10 d = −293 mV, 30 d = −330 mV, and 40 d = −361 mV).

3.1. Root and Nodule Distribution. In experiment 1, enhanced rooting (adventitious roots) and nodule formation at stem base between the water surface and soil level were observed in waterlogged plants beginning from day 20 of the treatment (Figures 1 and 2). Root DM weight in waterlogged plants was primarily distributed in shallower soil layers compared to the controls ($P < 0.05$). Throughout the study period, the total root DM weight of waterlogged plants was similar to that of the controls. When exposed to waterlogging treatment for 10, 20, and 30 days, 86.6%, 69.4%, and 77.8%, respectively, of the total root DM weight were found within the first 5 cm of the soil. In controls, 41.6% of the total root DM weight was distributed in the same area after 30 days of treatment.

The nodule DM weight in waterlogged plants was greater than that in the controls after 10, 20, and 30 days of treatment ($P < 0.01$). Nearly all of the nodules were found between 5 cm above and 5 cm below the soil surface.

In experiment 2, the total root DM weight in waterlogged plants was lower than that in the controls for the first 20 days of treatment. This was particularly emphasized in lateral roots (Figure 3). However, after 40 days of treatment, the total root DM weight in waterlogged plants was similar to that in the controls, because of the enhanced adventitious and lateral root development.

Total nodule DM weight was higher in waterlogged plants compared to the controls, particularly between day 5 and day 20 of the treatment ($P < 0.05$). However, after 40 days of treatment, the total DM weight of waterlogged plant nodules did not differ from that of the controls (Figure 4). The DM weight of nodules that formed on the taproot of waterlogged plants 5–20 days after the start of treatment was greater compared to the controls ($P < 0.05$). In both waterlogged plants and controls, the DM weight of taproot nodules was significantly greater than that of lateral root nodules 20 days after the start of the treatment ($P < 0.05$). However, after 40 days of treatment, the taproot nodule DM weight in the controls was significantly lower than the lateral root nodule DM weight ($P < 0.05$). After 40 days of treatment, the DM weight of the nodules that formed on the taproot, lateral roots, and stem base was similar in proportion. However, the DM weight of the lateral root nodules in waterlogged plants was significantly lower than that in the controls ($P < 0.05$).

The average DM weight of individual taproot nodules was higher in waterlogged plants than in the controls (Figure 5), particularly after 11–40 days of treatment ($P < 0.05$). Similarly, the average individual lateral root nodule DM weight in waterlogged plants was slightly higher than that in the controls. The average DM weight of basal stem nodules, found between the soil surface and water surface in waterlogged plants, was greater after 40 days of treatment than the average DM weight of the control taproot nodules ($P < 0.05$). Nodules on the adventitious roots of waterlogged plants showed lower DM weight than those on the lateral roots. After 40 days of treatment, the average DM weight of nodules formed on the taproot and stem base of waterlogged plants was significantly greater than the DM weight of nodules on the lateral and adventitious roots ($P < 0.05$).

3.2. Plant Growth. The DM weight of waterlogged plants in experiment 2 was lower than that of the controls for the first 20 days of treatment but higher compared to the controls after 40 days of treatment ($P < 0.05$) (Figure 6). The basal stem diameter of waterlogged plants was significantly larger than that of the controls after 2, 5, 11, and 40 days of treatment ($P < 0.05$; Figure 7).

3.3. Nitrogen Fixation. In experiment 2, the total TNA was significantly higher in waterlogged plants than in the controls after 5–11 days of treatment ($P < 0.05$). However, after 20 days of treatment, there was no significant difference in TNA between waterlogged plants and the controls (Figure 8). After 40 days of treatment, TNA in the above-ground parts of waterlogged plants was greater than that in the underground parts ($P < 0.05$).

In waterlogged plants, the SNA after 2–11 days of treatment was higher than that in the controls ($P < 0.05$; Figure 9). However, these values did not differ between groups after 20 and 40 days of treatment. In waterlogged plants, the above-ground SNA was significantly higher than the below ground SNA after 40 days of treatment ($P < 0.05$).

4. Discussion

Mesophytes, which have low tolerance to waterlogging, when grown in flooded soils exhibit a decrease in photosynthesis [42], reduced nutrient uptake [7–9], and inhibition of plant growth [10, 11]. In the present study, in the first 20 days of treatment, waterlogged American jointvetch plants were slightly smaller (Figure 6) than the controls, but the waterlogged plants were found to be slightly larger than the controls after 40 days of treatment. We have previously reported [35, 36] that the specific absorption rates of calcium and magnesium in plants subjected to 20 days of waterlogging were lower than those in the controls. However, calcium and magnesium absorption was greater in plants subjected to 20–30 days of waterlogging than in the controls [35, 36]. Net assimilation rate and photosynthate content were higher in waterlogged Glenn jointvetch than in the controls [35].

Plants grown in wet soils have shallow roots that are found primarily near the soil surface [6, 24–26]. In the

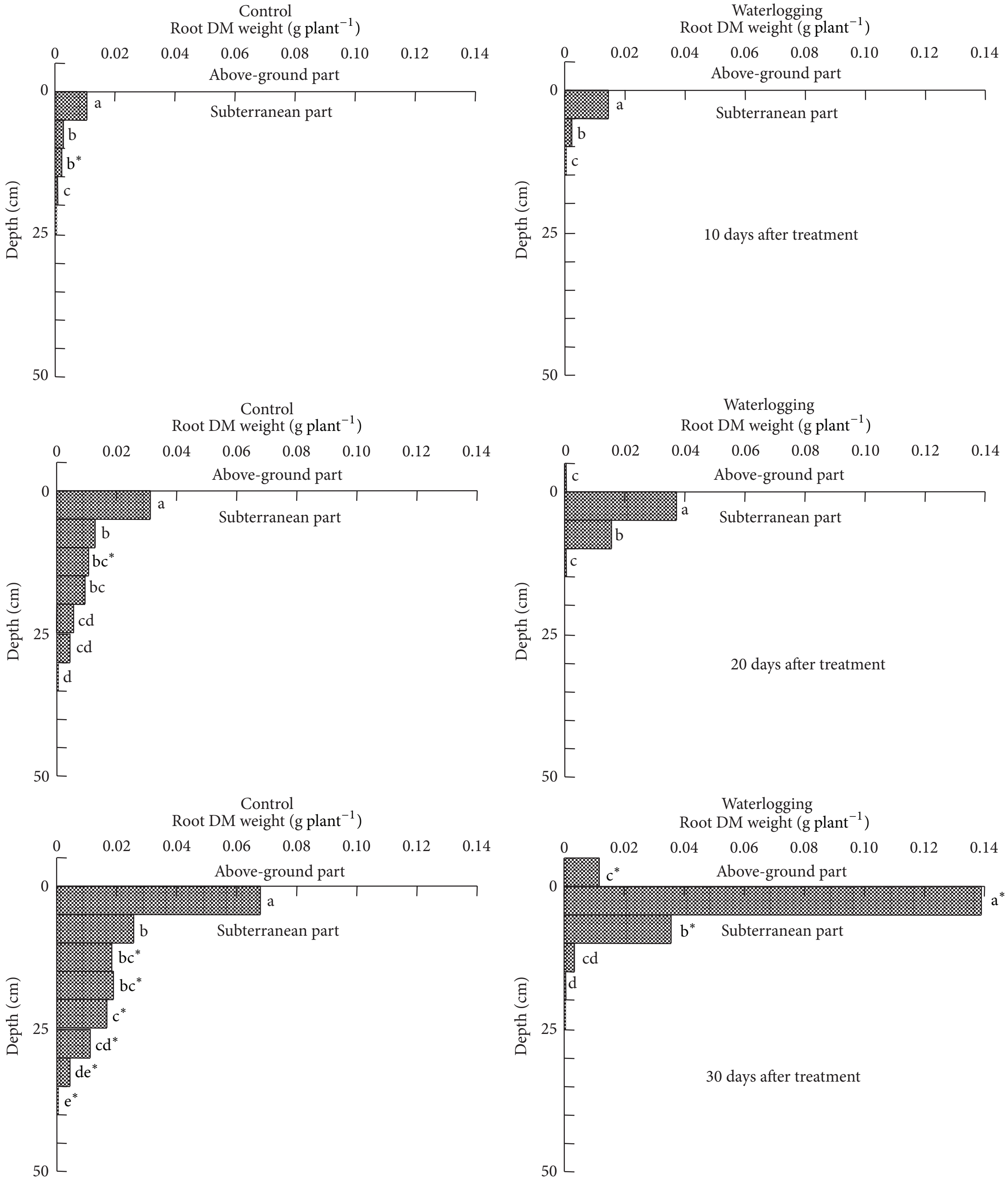

FIGURE 1: Distribution of roots from 10 to 30 days after treatment in experiment 1. Symbols with different letters within the same investigation date and treatment show significant differences ($P < 0.05$). *Significantly different ($P < 0.05$) between control and waterlogging treatment with the same root depth.

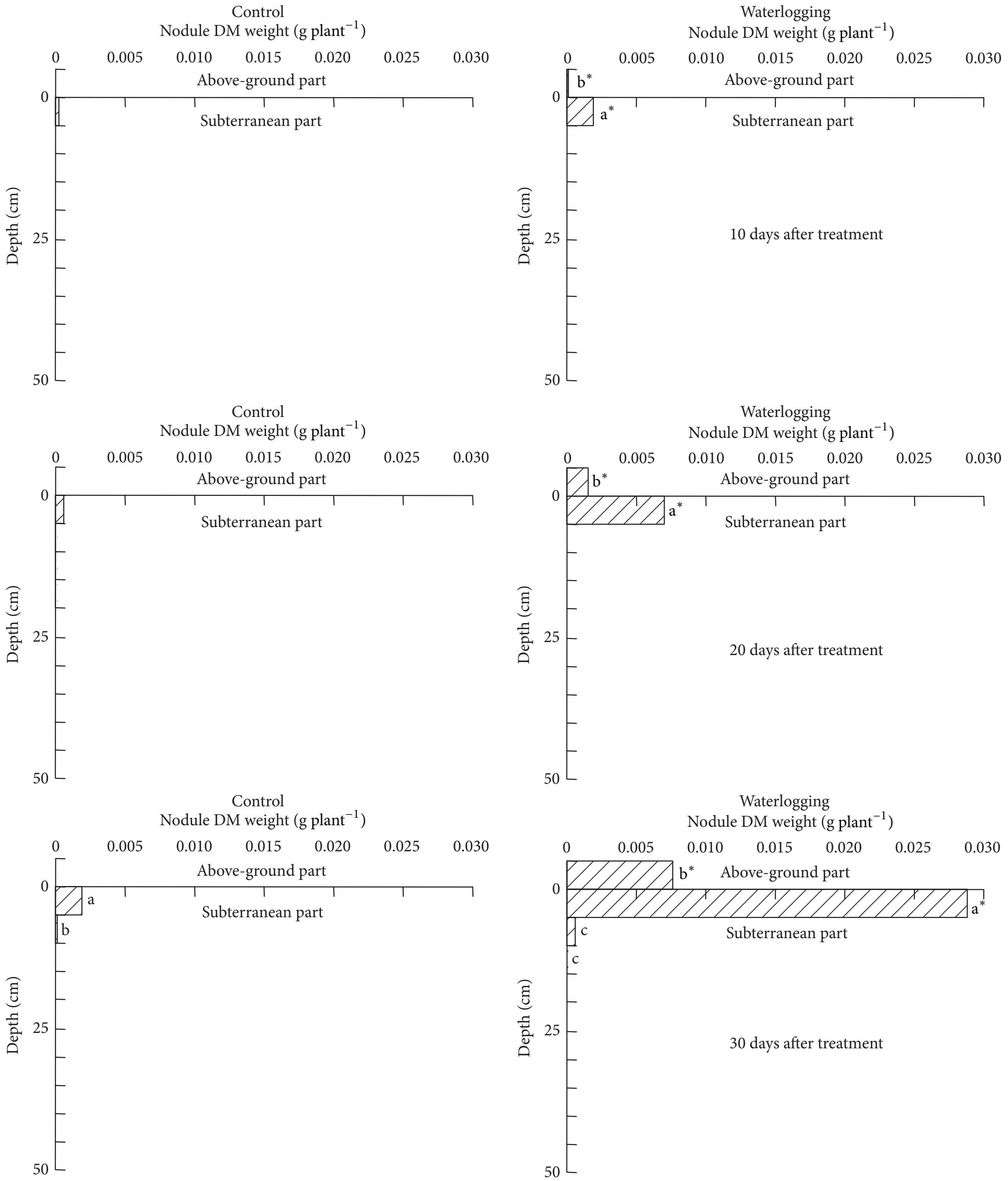

Figure 2: Distribution of nodules from 10 to 30 days after treatment in experiment 1. Symbols with different letters within the same investigation date and treatment show significant differences ($P < 0.05$). *Significantly different ($P < 0.05$) between control and waterlogging treatment with the same root depth.

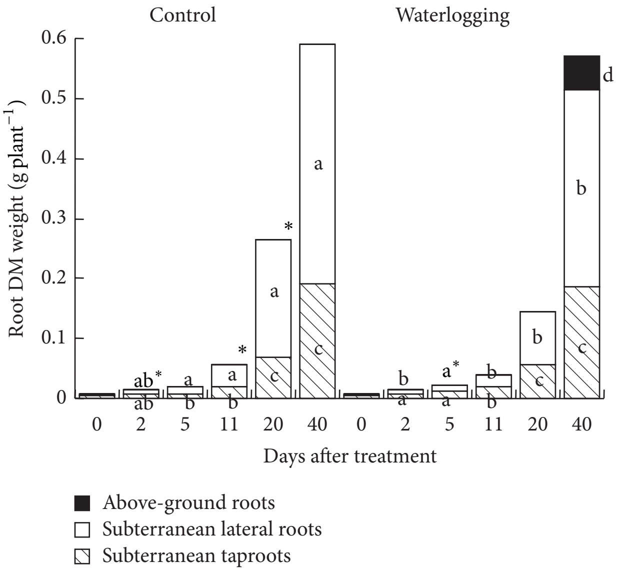

FIGURE 3: Changes in root dry matter (DM) weight in experiment 2. Symbols with different letters within the same investigation date and treatment show significant differences ($P < 0.05$). *Significant difference ($P < 0.05$) in the total root DM weight at the same day.

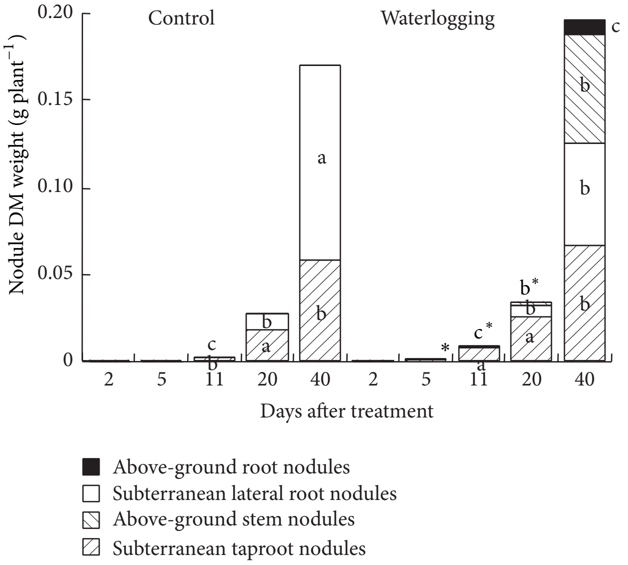

FIGURE 4: Changes in nodule DM weight in experiment 2. Symbols with different letters within the same investigation date and treatment show significant differences ($P < 0.05$). *Significant difference ($P < 0.05$) in the total nodule DM weight at the same day.

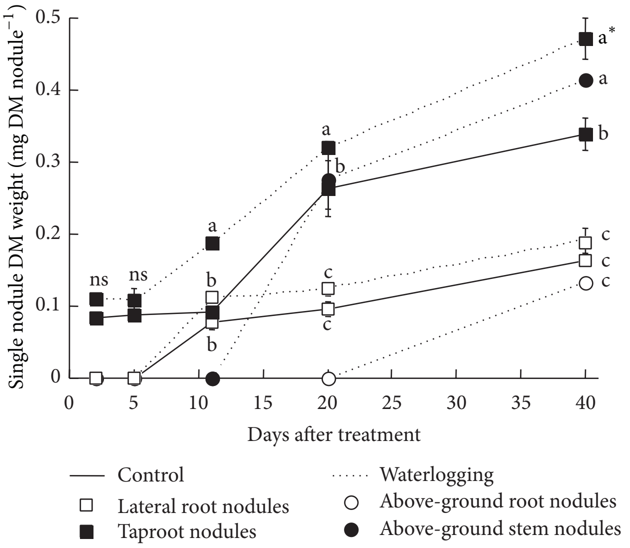

FIGURE 5: Changes in single nodule DM weight in experiment 2. *Symbols with different letters at each sampling day are significantly different at $P < 0.05$; ns: not significant. Error bars give ± one SE ($n = 3$).

present study, the roots of waterlogged plants were shallower than those of the controls (Figure 1), and the roots in both groups were distributed around the soil surface and in the area between the soil surface and water surface after 20–40 days of treatment (Figures 1 and 3). Plants with high tolerance to wet conditions form adventitious roots and secondary aerenchyma when subjected to waterlogging [12–17, 22, 43]. Secondary aerenchyma is found in waterlogged soybean plants [14, 15, 22]. However, although a basal stem thickening was detected from day 2 of waterlogging treatment (Figure 7), no secondary aerenchyma was observed in waterlogged American jointvetch, when observed under a stereoscopic microscope. When plants are exposed to waterlogging, ethylene biosynthesis in plants initiates the thickening of the stem base and the formation of the aerenchyma tissue [29, 44, 45]. It was reported that a swelling of the submerged portions of the stem base [27] and the appearance of hypertrophic lenticels [27, 28] also facilitated oxygen entry into the aerenchyma of the nearby adventitious roots [29]. The swelling is a consequence of cell expansion promoted by endogenous ethylene trapped by water in the submerged tissue [28, 29]. Under waterlogging conditions, aerenchymatous adventitious roots emerge from the shoot base to replace longer and deeper roots damaged or killed by anoxia [29, 30]. In maize, ethylene promotes the outgrowth of root primordia from the stem base [29]. The ability of some species to survive waterlogging was attributed to shallow rooting [31], and those species can reorientate root extension to a more horizontal or even upright direction and thus towards zones where oxygen may be more readily available [29]. Developing an adventitious root system is an important strategy that plants use to avoid anoxia [29, 31]. We believe that the thickening of the stem base starts immediately after the treatment, and the root distribution around the soil surface and in the area between the soil surface and water surface is the result of the tolerance that the American jointvetch has developed to waterlogging.

Leguminous plants and rhizobia maintain a symbiotic relationship, where the rhizobium cannot bring about nodulation and nitrogen fixation without a supply of photosynthates from the host plants. Waterlogging-induced anaerobic conditions in the soil are harmful to the formation and function of nodules in several legume species [15, 19, 20,

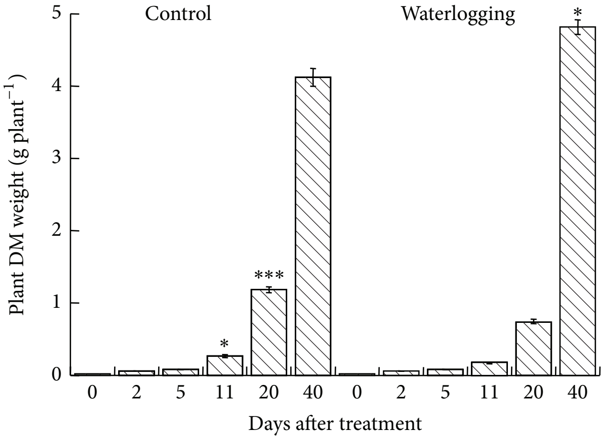

Figure 6: Changes in plant DM weight in experiments 2. *** and *Significantly different ($P < 0.001$ and $P < 0.05$) between control and waterlogging treatment at the same sampling day. Error bars give ± one SE ($n = 3$).

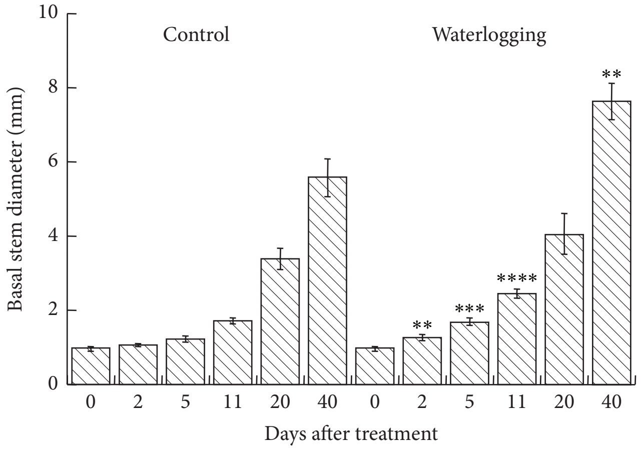

Figure 7: Changes in basal stem diameter in experiment 2. ****, ***, **, and *Significantly different ($P < 0.0001$, $P < 0.001$, $P < 0.01$, and $P < 0.05$) between control and waterlogging treatment at the same sampling day. Error bars give ± one SE ($n = 3$).

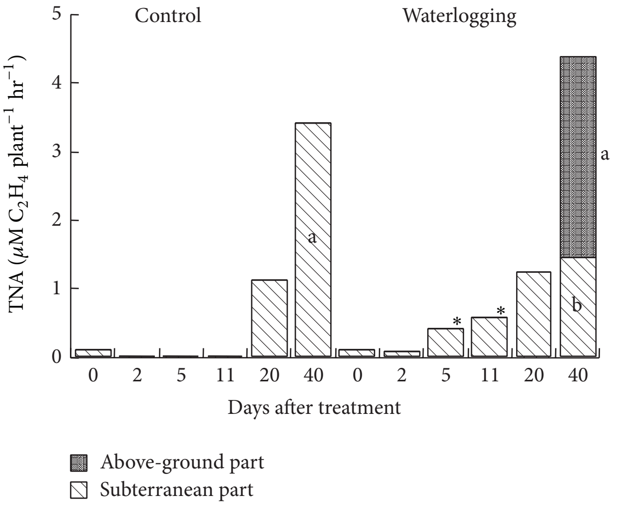

Figure 8: Changes in total nitrogenase activity (TNA) in experiment 2. Symbols with different letters within the same investigation date and treatment show significant differences ($P < 0.05$). *Significant difference ($P < 0.05$) in the total TNA at the same day.

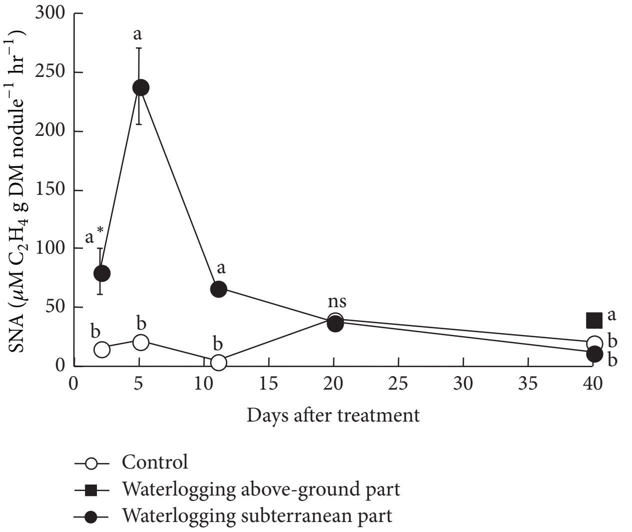

Figure 9: Changes in specific nitrogenase activity (SNA) in experiment 2. *Symbols with different letters at each sampling day are significantly different at $P < 0.05$; ns: not significant. Error bars give ± one SE ($n = 3$).

24, 46]. A decreased nitrogen accumulation observed under waterlogging conditions is due, in part, to reduced nodulation and decreased nitrogenase activity [47, 48]. Inhibition of nodulation by waterlogging has been reported for several legume species [48, 49] and is attributable to the adverse effects of waterlogging on rhizobial populations, decreased frequency of root infection [11], and suppression of root growth and root hair development [49, 50]. The anaerobic conditions may reduce the energy supply available for the formation of nodules [51, 52]. However, in our experiment, TNA, SNA, and nodule weights in plants subjected to waterlogging treatments were similar to or higher than those in the controls (Figures 2, 4, 8, and 9). The waterlogged plants had larger individual nodules (Figure 5) located around the soil surface (0–5 cm below the soil surface) and in the area between the soil surface and water surface (Figures 2 and 4) as well as higher TNA in the above-ground plant parts (Figure 8) than the controls after 20 days of treatment. The SNA 2 days after waterlogging treatment showed the highest value during the experimental period (Figure 9). We were not able to explain this pathogenesis. Highly wet-tolerant plants develop aerenchyma tissue in the roots and transport gases (e.g., O_2 and N_2) from foliage to roots when exposed to waterlogging [53, 54]. These plants maintain a high level of nodule formation and nitrogen fixation activity when waterlogged [6, 15, 53–55]. Albrecht et al. [39] reported that waterlogged *A. americana* showed similar nitrogen fixation activity to the controls and hypothesized that this was because the gases for nitrogen fixation were transported through an aerenchyma in waterlogged plants. Loureiro et al. [56], using a transmission electron microscopy method, observed aerenchyma and spongy tissue at the base of the nodule that connected both stem and root nodules to a large intercellular space in the cortex of *Aeschynomene fluminensis*. Yoshida [54] reported that the gases necessary for nitrogen fixation were mainly transferred to the root system through an aerenchyma in waterlogged *Aeschynomene indica*. Tobisa et al. [37], in their greenhouse experiment of 30 days of waterlogging treatment of the same genus *Aeschynomene* (three *A. americana* strains and two *Aeschynomene villosa* strains), reported that nitrogen fixation activity and nodule DM weight in the waterlogged plants were higher than those in the control group starting from 7 days after germination. In the present study, we observed similar nitrogen fixation activity in waterlogged and control *Aeschynomene americana*. These results indicate that the delay in growth in the first 20 days of waterlogging treatment is caused by the initial employment of the photosynthate for aerenchyma formation, nodulation, and nitrogen fixation. As shown in previous studies, after 20 d of treatment, the plants acquire tolerance to waterlogging, and the growth resumes [35, 37]. In addition, in the present study, the lateral root nodule DM weight in waterlogged plants after 40 days of treatment became lower than that in the control (Figure 4). This suggests that the rate of aerenchyma formation in lateral root is slower compared to the aboveground stem, and the nodules that are formed in the aboveground stem develop more aerenchyma than the lateral roots. Additionally, these results indicate that the nodules formed in the aboveground stem promote TNA after 20 days of waterlogging treatment.

Stem nodulation has been reported in 21 species of the genus *Aeschynomene*, three species of the genus *Sesbania*, and *Neptunia oleracea* [57]. Ladha et al. [57] divided stem-nodulating legumes into three groups. Most of *Aeschynomene* species that have stems with visible nodules/root primordia but form nodules mainly on the submerged stems are included in group 2. *Aeschynomene americana* was not included in that study [56], but, based on the characteristics of the stem nodule formation, we believe that it should belong to this group. Stem nodulation is more important than the root nodulation for plant nitrogen fixation and dual (stem and root) nodulation results in higher rate of nitrogen fixation than single (root) nodulation [57]. In *Sesbania rostrata*, stem nodules appear at approximately 4 weeks of age and account for more than 80% of total acetylene reduction activity, which further increases to almost 100% between 4 and 7 weeks of age [58]. In this study, the proportion of DM weight of above-ground nodules and TNA increased with increasing waterlogging period; this result is in agreement with those of previous studies [57, 58].

Additionally, we found that waterlogged American jointvetch plants have basal stem thickening from 2 days of waterlogging treatment and roots with nodules that are distributed both around the soil surface and in the area between the soil surface and water surface after 20 days of waterlogging. The plants maintained high nitrogenase activity and net assimilation rate, which in turn increased the above-ground nodule DM weight, TNA, growth, and yield. However, in our study, hypocotyl (secondary) aerenchyma was not observed in the waterlogged plants. Therefore, future studies are needed to investigate the aerenchyma development and metabolic activity in the roots and nodules in waterlogged American jointvetch plants.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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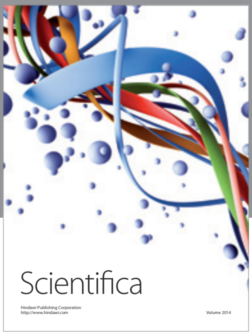
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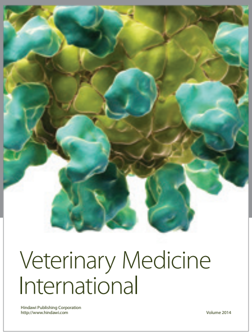
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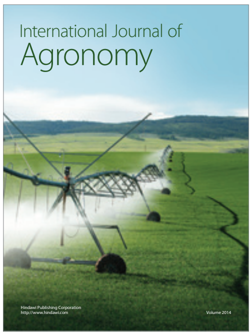
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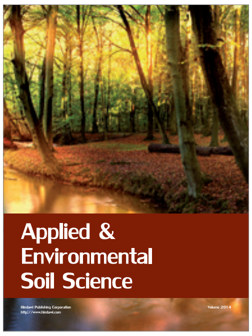
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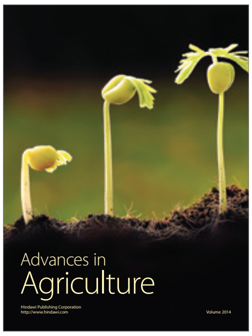
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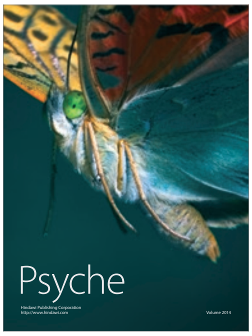
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