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Research Article

Species Specific Responses to Age on Nodule Formation, Seedling Growth, and Biomass Production of *Acacia auriculiformis* at Nursery Stage

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Nodulation responses of leguminous trees are very important for intercropping to reduce reliance on artificial nitrogen input through nitrogen fixation in agroforestry system. This study was carried out to evaluate the status of nodulation (i.e., the number of nodules and their shape and size) in root and biomass production of plant growth parameters (i.e., number of leaves, shoot height, root biomass, and shoot biomass) of *A. auriculiformis* seedlings. The assessment was conducted 60 days after seed germination. The study revealed significant differences in nodule number per seedling, leaf number per seedling, shoot height, and biomass accumulation (both green and oven dry weight) with seedling age ($p < 0.05$). The study also revealed significant correlation among the variables of nodulation responses and biomass production. The results obtained using principal component analysis (PCA) justified correlation matrix of nodulation responses and biomass production of this species. The PCA showed that root biomass per seedling, leaf number per seedling, nodule number per seedling, shoot height, age of seedling, and shoot biomass per seedling were clustered with PC1 (with an eigenvalue of 5.59) and root shoot ratios were clustered with PC2 (with an eigenvalue of 1.82). Our study justified that shoot height may be an important determinant of nodule formation of *A. auriculiformis*.

1. Introduction

In tropical countries, leguminous tree species are considered prospective agroforestry components not only for soil enrichment, fuel, and forage [1] but also for a fundamental food supplier for tropical livestock [2]. Leguminous trees are extensively used for intercropping to reduce dependency on artificial N_2 input through growing symbiotic nitrogen fixation as sustainable agroforestry system both in the tropics and in temperate regions [3–8]. This system provides significant benefits to increase land use competency [9] and for harmonizing diet with legumes, the prime protein (energy) source for wild animals, livestock, and also for human beings [10]. Nitrogen fixing trees play an important role in establishment and improvement of N_2 deficit soil. That is why nitrogen fixing trees are considered as perfect species for agroforestry and reforestation of degraded land [11, 12]. Leguminous trees used in agroforestry transfer nitrogen to the associate agricultural crops and stimulate their growth [6, 8, 13–16]. Symbiotic relationship between agricultural crops and leguminous trees in agroforestry systems mainly concentrates on N_2 fixation, attainment, and transformation [4]. The present situation of forests in Bangladesh is incapable of fulfilling the needs and aspirations of people for forest products because of over population and exploitation and intensive land use changes and maybe because of climate change. Bangladesh government is concerned about overcoming this dilemma in restoring forest lands and has given priority for growing trees on farms and homesteads. Government's as well as private sector's initiatives in tree plantation programmes have achieved momentum throughout the country, especially on degraded fallow lands, agroforestry, and homesteads [17]. Farmers' choice for species selection of agroforestry and homesteads cultivation should be encouraged [18–20]. *Acacia auriculiformis* is widely grown in agroforestry, roadside plantation, and home garden in Bangladesh. *A. auriculiformis* is a member of Fabaceae family, which is a multipurpose, leguminous tree species widely distributed in many tropical countries of South and Southeast Asia, Latin America, and Africa [21]. It is an attractive fast growing tree species [22, 23], planted for fuel wood production, nitrogen fixation, and erosion control [24]. It is also planted as shade bearer, ornamental, sources of tannin, and pulp producing plant in the world [24]. For its nitrogen fixing ability, rapid growth, and tolerance of infertile soil, it is considered as a useful species for rehabilitation of fallow lands [17, 25]. The environmental and soil condition of the country improve through plantation of this species. Huda et al. [26] and Uddin et al. [27] studied the effects of NPK fertilizers on nodule formation of some agroforestry species in Bangladesh. Azad et al. [20] conducted nodulation responses and biomass production of *L. leucocephala* and *A. saman*. However, information on the response of nodulation and biomass production of *A. auriculiformis* is not available. Thus, we made an endeavor to investigate nodulation responses and biomass production in relation to age of *A. auriculiformis* at the nursery stage. The objectives of this study are as follows: (i) to explore the differences in nodule formation and biomass production in relation to age and (ii) to determine the practical relationship between the variables of nodule formation and biomass production at early stage of *A. auriculiformis*.

2. Materials and Methods

2.1. Study Area. The study was conducted at Khulna University, Bangladesh. Seed germination and seedling establishment were done at the nursery of Forestry and Wood Technology Discipline, Khulna University, Bangladesh, and nodule assessment and biomass measurement were carried out at Forestry and Wood Technology lab, Khulna University, Bangladesh, which is located about 4 m above sea level. The geographic position of the study area is between 22.802°N latitude and 89.533°E longitude. The climate of this area is recognized as subtropical in nature, very much similar to the other regions of the country particularly in winter, summer, and monsoon. Winter starts in November and ends in February, summer starts in March and continues up to mid-June, and the remaining period is occupied by monsoon the distinguishing season. The temperature variation in winter is very low (7 to 12°C) but it rises from 25 to 32°C in summer. Occasionally it increases up to 40°C [28]. Average air temperature recorded was 21–34°C and the relative humidity was 69–86% during the experimental period.

2.2. Plant Material and Design of Experiment. Seeds were collected directly from roadside plantation and private woodlot and then dried under the sun for three days to reduced moisture content. The collected seeds were then separated from the pods and only healthy seeds were used for germination. Each seed was placed in one poly bag (10 cm × 15 cm). The seeds were treated with hot water (80°C for 10 min) [18] prior to putting the seeds in poly bags. A mixture of cow dung, TSP, and forest soil (2 : 1 : 3) was used as germination media [20]. Urea fertilizer was used 25–30 days after seed germination. Ripcord solution and tuin powder were applied together to prevent the seedlings from insect attack. Watering and weeding were done when necessary. Eight hundred and forty seedlings were used for the experiment. Nodulation assessment was started 60 days after seed germination and continued monthly for the next six months. Every month twenty-five seedlings were randomly selected for nodulation assessment and biomass study. The poly bags were removed from the selected seedlings and submersed under water for 2 h to loosen the soil which made it simple to eliminate the soil from the seedlings' root and helped to maintain the nodules undamaged. The root of the selected seedlings was washed out carefully with clean water to confirm that no mud was attached to root system. The root and shoot of each seedling were separated by using a sharp knife. The shape, size, numbers of nodules, numbers of leaves, and height of shoot (cm) were recorded for the assessment of nodulation responses. The green and oven dry weight (g) of root and shoot were measured for the assessment of biomass production. The forms and color of the nodules were also recognized and recorded. Nodule and leaf number were counted manually. Electronic digital caliper (accuracy ± 0.02 mm) was used for measuring nodule size. Shoot height was measured by using a centimeter scale. An electronic balance was used for measuring of green and oven dry weight of root and shoot of the seedlings. The root and shoot were placed separately in an oven at 80°C for 24 h to take first measurement of oven dry weight. Data were recorded every one-hour interval after the first measurement until maintaining constant weight.

2.3. Data Processing and Analysis. The collected data were standardized to get standard Z score to ensure that different variables with different units are compared. The following equation was used for data standardization:

$$Z = \frac{X - \overline{X}}{S}, \tag{1}$$

where $\overline{X}$ is the sample mean, S is standard deviation, and Z is the distance between the raw values and the population mean. Z score can be positive or negative. Negative value means values below the mean value and positive indicates being above the mean values. The standardized data were analyzed by using Microsoft Excel 2007, Statistica (version 10), and PAST [29] software. One-way analysis of variance (ANOVA) was conducted to identify the difference in nodule formation with seedling age. Analysis of variance was also carried out to identify significant difference in leaf number, shoot height, root biomass, shoot biomass, and root shoot ratio (both green and oven dry weight) with seedling age. The Mann-Whitney test was also conducted to identify significant difference between root biomass and shoot biomass of seedlings. The relationships between the variables of nodulation responses and biomass production were established through correlation coefficients (r) to specify the strength and direction of relationship between variables. Principal component analysis (PCA) was conducted to find out important correlated components of underlying variables of nodulation responses and biomass production of *A. auriculiformis* seedlings.

Table 1: General characteristic of nodules of *A. auriculiformis* seedlings.

Age (days)	Diameter (mm)	Form	Shape	Color
60	2.7–4.5	Single and aggregate	Elongated	Whitish
90	3.8–4.5	Single and aggregate	Elongated	Whitish
120	6.9–9.5	Single and aggregate	Elongated	Whitish
150	7.7–13.3	Single and aggregate	Elongated	Whitish
180	5.7–19.3	Single and aggregate	Elongated	Whitish

Table 2: Summary of nodulation responses of different particulars of *A. auriculiformis* with age. Data shows mean values ± SE. The same letter in the same row indicates no significant differences.

Particulars	60 days	90 days	120 days	150 days	180 days	p value
NN	8.8 ± 1.96^{c}	9.4 ± 2.32^{c}	31.1 ± 2.6^{b}	34.6 ± 2.8^{ab}	44.4 ± 3.78^{a}	0.000
LN	14 ± 2.78^{d}	35.6 ± 7.4^{bc}	38.2 ± 2.6^{c}	46.4 ± 5.16^{ab}	59.8 ± 4.36^{a}	0.000
R/S (gr)	0.156 ± 0.013^{c}	0.318 ± 0.045^{ab}	0.36 ± 0.04^{a}	0.25 ± 0.02^{bc}	0.27 ± 0.03^{ab}	0.003
R/S (od)	0.29 ± 0.02^{c}	0.20 ± 0.06^{ab}	0.22 ± 0.04^{a}	0.35 ± 0.01^{c}	0.37 ± 0.02^{bc}	0.001
RBM	0.32 ± 0.11^{d}	1.29 ± 0.32^{c}	2.68 ± 0.11^{c}	5.88 ± 0.58^{b}	8.2 ± 0.61^{a}	0.001
SBM	1.09 ± 0.16^{e}	6.6 ± 0.49^{d}	12.3 ± 1.15^{c}	16.6 ± 1.01^{b}	22.4 ± 1.9^{a}	0.003
Height	21.2 ± 1.02^{d}	24.2 ± 3.92^{c}	26.9 ± 2.55^{bc}	27.8 ± 2.65^{ab}	36 ± 3.74^{a}	0.000

Note: NN: nodule number per seedling, LN: leaf number per seedling, R/S (gr): root shoot ratio of green weight, R/S (od): root shoot ratio of oven dry weight, RBM: root biomass per seedling, and SBM: shoot biomass per seedling.

Table 3: Matrix of correlation coefficients (r) of different variables of nodulation responses and biomass production of *A. auriculiformis* seedlings.

	Age	NN	LN	SH	RBM	SBM	R/S (gr)	R/S (od)
Age	**1**							
NN	0.96^{**}	1						
LN	0.94^{*}	0.81	1					
SH	0.95^{*}	0.90^{*}	0.92^{*}	1				
RBM	0.98^{**}	0.93^{*}	0.94^{*}	0.94^{*}	1			
SBM	0.99^{**}	0.96^{**}	0.94^{*}	0.95^{*}	0.97^{**}	1		
R/S (gr)	0.31	0.32	0.32	0.28	0.12	0.34	1	
R/S (od)	0.62	0.54	0.54	0.57	0.76	0.59	−0.54	1

Note: NN: nodule number per seedling, LN: leaf number per seedling, SH: shoot height, RBM: root biomass per seedling, SBM: shoot biomass per seedling, R/S (gr): root shoot ratio of green weight, and R/S (od): root shoot ratio of oven dry weight. $*$ indicates statistical significance at $p < 0.05$ and $**$ indicates significance at $p < 0.01$.

3. Results

3.1. Nodule Formation. The seedlings of *A. auriculiformis* possessed nodules in both single and aggregate forms (Table 1). The shape and color of the nodules were elongate and whitish, respectively. The study that revealed number of nodules per seedling increased with age (Table 2). The rate of increase in nodule number was relatively slow up to 90 days; then it increased tremendously for the next 30 days and maintained a steady rate for another 30 days and increased again sharply from 150th day to 180th day. One-way analysis of variance (ANOVA) showed significant difference ($p < 0.001$) in nodule number per seedling with increasing age (Table 2).

3.2. Seedling Growth. The study disclosed very fast seedling growth (height growth) at nursery stages. Height growth was significantly ($p < 0.001$) increased with age of the seedlings. The study also disclosed significant increase in number of leaves per seedling ($p < 0.001$) with age (Table 2).

3.3. Biomass Production. The study revealed significant difference ($p < 0.001$) in root and shoot biomass with increasing seedling age. The Mann-Whitney test also revealed significant difference ($p < 0.01$) between shoot biomass and root biomass (results not shown). Our results also disclosed significant difference in root shoot ratio (both oven dry and green weight) with age.

3.4. Relationship between Different Variables of Nodule Formation and Biomass Production. There was a strong positive relationship between the variables of nodulation responses and biomass production (Table 3). The number of nodules increased very slowly up to 90 days and then

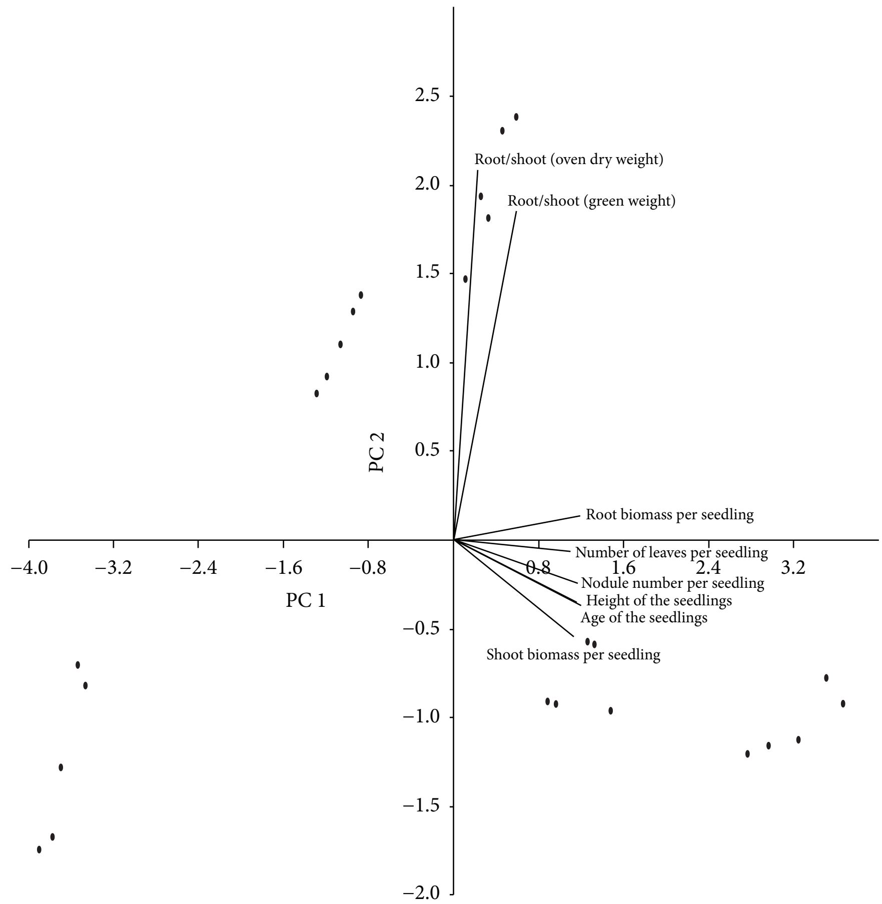

Figure 1: Principal component ordination of nodulation responses and biomass production of *A. auriculiformis*. The horizontal axis of the ordination plot shows the first principal component (PC1) with an eigenvalue of 5.59, explaining 69.9% of total variation in nodule formation and biomass production; the vertical axis shows the second principal component (PC2) with an eigenvalue of 1.82, explaining 22.9% of total variation of nodule formation and biomass production.

increased sharply (Table 2). The correlation matrix (Table 3) revealed significant positive correlation in nodule number per seedling, leaf number per seedling, shoot height, root biomass, and shoot biomass with age of the seedlings. The correlation matrix also revealed significant positive relationship of shoot height, root biomass, and shoot biomass with nodule number and leaf number per seedling. Shoot height also showed significantly positive correlation with root biomass and shoot biomass. Root biomass also showed significant correlation with shoot biomass.

Principal component analysis (PCA) of nodule formation and biomass production explained 69.9 and 22.9% of variance with PC1 and PC2, respectively, with a big difference in eigenvalues between PC1 (5.59) and PC2 (1.82). Root biomass per seedling, number of leaves per seedling, nodule number per seedling, height and age of the seedling, and shoot biomass per seedling were clustered together (Figure 1) with PC1 indicating close relationship between the variables which was reflected in correlation matrix (Table 3). On the other hand, root shoot ratio (both oven dry and green weight) was completely separated from other variables and clustered together with PC2 (Figure 1) which was also justified with correlation matrix (Table 3).

4. Discussion

Seedlings of different fast growing tree species of agroforestry component possess nodules in different shapes and forms. The external shape of nodules changes during the growth period of the seedlings. Azad et al. [20] documented both single and aggregate form of nodules in *A. saman* and *L. leucocephala*. This study investigated whitish elongated form of nodules in *Acacia auriculiformis* seedlings. *A. auriculiformis* possess more aggregate form of nodules in six-month-old seedlings. Azad et al. [20] found more aggregated nodules in *A. saman* than in *L. leucocephala.* Khan et al. [30] also found single and aggregated form of nodules of *A. saman* and *L. leucocephala* three months after planting the seedlings. Qadri and Mahmood [31] found single nodules and cluster of nodules on main and lateral roots in *S. saman*. They opined that bacteria entered into root system via root hair and formed infected thread. Azad et al. [20] reported that young

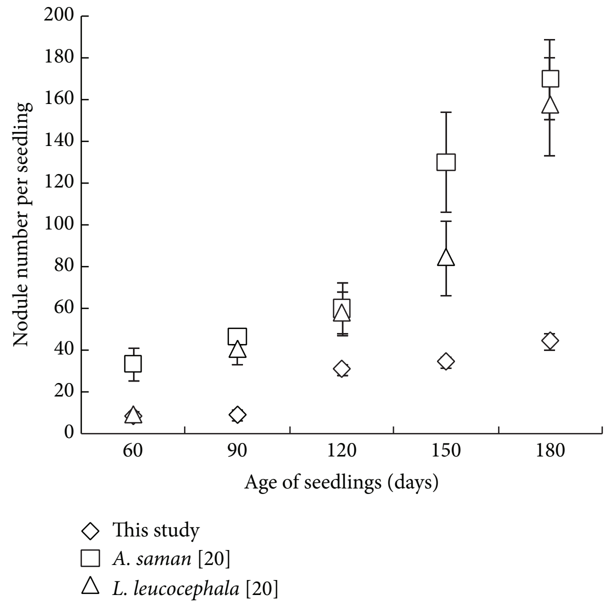

Figure 2: A comparative analysis with other studies of nodule formation with seedling age at early stages. Vertical bar indicates mean values ± SE.

nodules of *A. saman* were spherical in shape whereas old-age nodules were elongated. Aggregation of nodules increased gradually over time in early stage of seedling growth in some leguminous tree species [20]. Bacteria may invade more intensively over time and, thereby, colonize nodules in the root system. Age of the seedlings influences nodule formation, nodule biomass, and seedlings growth at early stages. Mrema et al. [32] and Azad et al. [20] found very much similar fashion of nodulation responses of *L. leucocephala* but they possess greater number of nodules and better seedling growth (seedlings height) than *A. auriculiformis* (Figures 2 and 3), considering nodule biomass of *A. auriculiformis* responded in between *L. leucocephala and A. saman* (Figures 2 and 3). Mrema et al. [32] documented the highest nitrogen activity in mature nodules and mature nodules contained a greater number of bacteroids compared to juvenile nodules of *L. leucocephala*. Azad et al. [20] also found higher nodule growth (number of nodules, nodule biomass) of *A. saman* and *L. leucocephala* in six-month-old seedlings (Figures 2 and 3). Nodule formation of *A. auriculiformis* exhibited significant ($p < 0.01$) correlation with age of the seedlings. Nodule number increased very slowly up to three months and then increased sharply and kept steady for another three months which indicates root development took place at the first few months and thereby developed nodules in the root system. Azad et al. [20] carried out a similar study on nodulation responses and biomass production of *L. leucocephala* and *A. saman* and found similar correlation between nodule number and age of the seedlings which indicates that age of seedlings is a determinant of nodule formation. Our results also revealed significant correlation between nodule number and shoot height (seedling growth) ($p < 0.05$), nodule number and root biomass ($p < 0.05$), and nodule number and shoot biomass ($p < 0.01$) (Table 3) which also indicate that shoot height, root biomass, and shoot biomass are also the determinants of nodule formation.

PCA demonstrates separate groupings of nodulation and biomass characteristics. PCA provides an illustration of relationships between the characteristics that are the most important key in defining the variables. Root biomass per seedling, leaf number per seedling, nodule number per seedling, height of seedlings, age of seedlings, and shoot biomass per seedling were clustered with PC1 (Figure 1), and thus correlation matrix is justified with PCA.

Azad et al. [20] also explained that age of seedlings, shoot height, leaf number, root biomass, and shoot biomass were the determinants of nodule formation of *L. leucocephala* and *A. saman* seedling at early stages. Among the determinants of nodule formation, shoot height was always bigger in *L. leucocephala* and *A. saman* seedlings compared to those of *A. auriculiformis* (Figure 3). At the same time nodule number of *L. leucocephala* and *A. saman* was also higher compared to *A. auriculiformis* (Figure 2) indicating that shoot height is a distinguishing determinant of nodule formation of those species. Nygren and Cruz [33] recommended that aboveground biomass influences nodule formation of leguminous tree species. Nygren and Ramírez [34] also documented production and turnover of nitrogen fixing nodules in comparison to foliage growth of *Erythrina poeppigiana* (Leguminosae) trees. Datta and Das [35] experimented to evaluate nodulation and its effects on biomass of a number of agroforestry leguminous tree species and documented a positive correlation between nodule biomass and shoot biomass. Robson et al. [36] and Jakobsen [37] also noticed

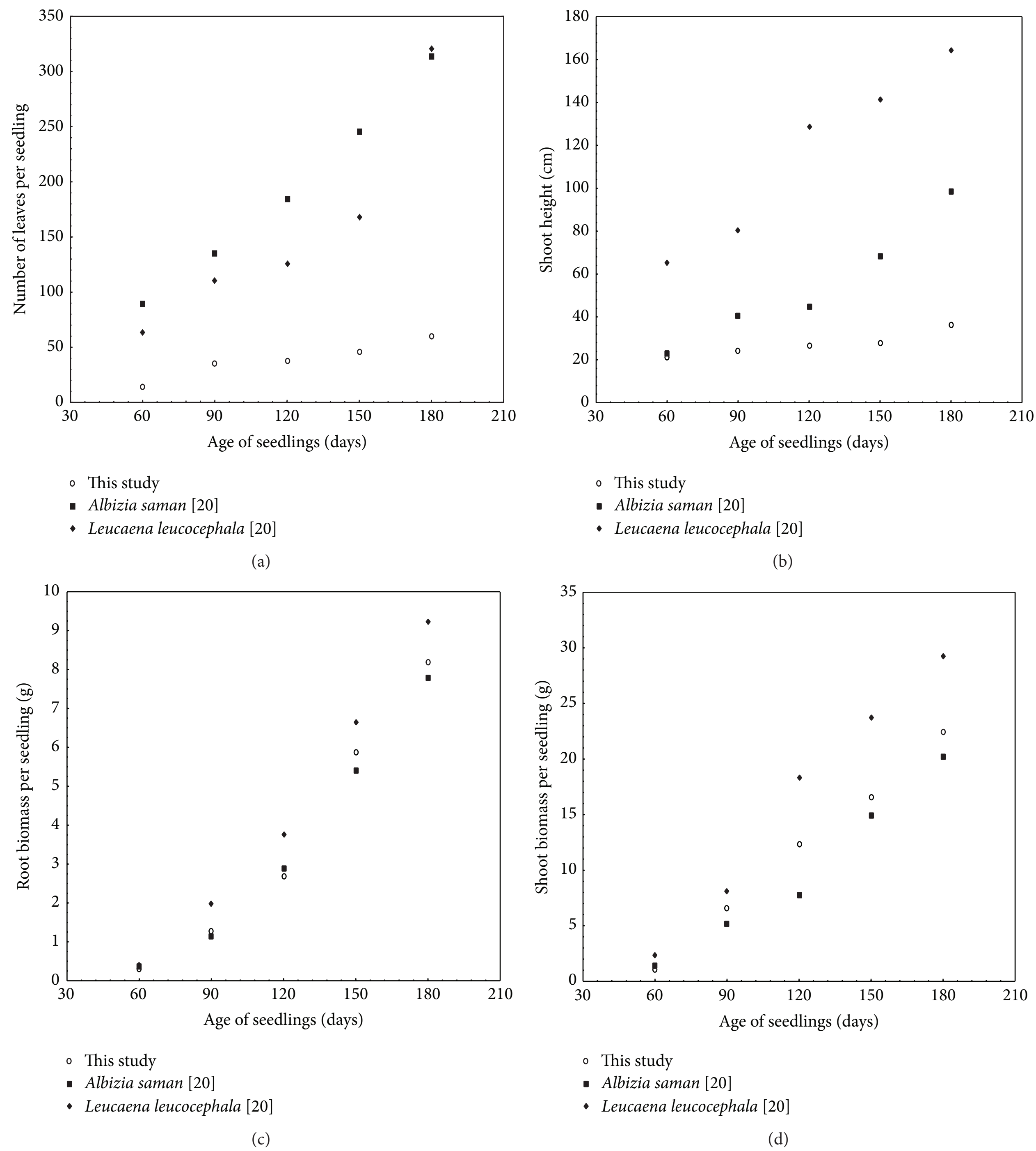

FIGURE 3: Comparisons of seedling growth and biomass production in relation to seedling age with other studies: (a) number of leaves per seedling, (b) shoot height (cm), (c) root biomass per seedling (g), and (d) shoot biomass per seedling (g).

a relationship between the nodule number and shoot growth of leguminous tree species.

5. Conclusions

Nodulation responses and biomass accumulation in root and shoot of the seedlings may depend on a variety of factors, such as fertilizer input from outside, water uses, and other external factors. It can be species oriented but from our study we can conclude that nodule formation in general increases with seedling age at the early stages of development of agroforestry species, such as *A. auriculiformis.* From this study, we can also infer that different variables of nodule formation and biomass production (root biomass and shoot biomass) are correlated with each other but shoot height can be an important indicator for nodule formation.

Competing Interests

The authors declared no conflict of interests concerning the publication of this paper.

Authors' Contributions

Experimental design, paper writing, and comprehensive data analysis were done by Md. Salim Azad. A partial data analysis and data collection was done by Md. Mehedi Hasan Sumon.

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