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ORIGINAL ARTICLE

Study Growth and N_2-fixation of *Alnus subcordata* seedlings inoculated with different Frankia crushed nodules

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ABSTRACT
The growth and nitrogen fixation of Caucasian alder (Alnus subcordata C.A.Mey) that inoculated with Frankia were investigated in pots filled with sterilized sand. The Frankia suspensions prepared from root nodules of A. subcordata that isolated from 25 different sites of Hyrcanian forest, northern Iran. The seedling growth, N_2-fixation and nodulation were measured 10 weeks after inoculation. Dry weight of shoots, roots and nodules, and N content ofthe seedlings inoculated with Frankia were higher than those without inoculation. The greatest impact on the nitrogen xing ability, microbial inoculation effects and plant biomass was observed in seedlings inoculated with AS50 Frankia crushed nodules compared with other treatments. The results of these experiments showed that the Frankia crushed nodules could improve the growth and nitrogen xation of A. subcordata. Thus, selection true sources of inoculums that have a considerable influence to A. subcordata and optimizing the sustainable production of these inoculums is needed.
Key word: *Alnus subcordata, Frankia, Growth, N_2-fixation*

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INTRODUCTION

Actinorhizal plants are the pioneer plants on N-poor soils. They used for afforestation [1], reclamation of degraded lands [2,3], soil amelioration [4] and short rotation forestry [5].Their capability to supply the majority of their N requirements via N_2-fixing symbiosis with Frankia ensures they are sensible applicants for improvement as sustainable plants for landscaping. From the above 200 taxa recognized to form actinorhizal symbiosis, species within the genus *Alnus*, demonstrate the greatest rates of N_2 -fixation and they are merit for ornamental use. *Alnus subcordata* CA Meyer is endemic to the forests of northern Iran. This species is one of the fast growing and valuable native species of the Hyrcanian forests. According to the inventory of the northern forest of Iran, *A. Subcordata* is the fourth species in Hyrcanian forests and has 7.75% and 8.6% of the total volume of timber species in northern Iran and Guilan forests, respectively. Therefore, according to the economic and ecological importance of this species, identifying methods to increase adaptability, stability and higher yield are essential.

In forest ecosystems, nitrogen is the major determining factor for the growth of trees. The nitrogen-fixing microorganisms can play an important role in these ecosystems. Earlier investigations have shown that significant amounts of nitrogen can be fixed by the Alnus-Frankia symbiosis. Input of fixed nitrogen can improve seedling growth, especially in nitrogen-deficient soils [6]. If attractive actinorhizal species can be selected and cultural methods developed to maximize the benefit of their N_2-fixing symbiosis, growers should be able to reduce N input during production. The optimal use of actinorhizal plants includes improvement of the symbiosis through the selection of superior genotypes of both partners. Glass-house experiments have shown considerable variation in the effectiveness of Frankia strains for nodulation and nitrogen fixation in plants such as alders [7, 8], whether originating from soil inoculums [9], or from crushed nodule preparations [10,11].The chance to enhance symbiotic fixation of nitrogen in actinorhizal plants by endophyte selection has arisen relatively recently, with the development of novel techniques and isolation of new Frankia sources. The utilization of crushed nodule preparations as inoculums has a practical value for nurserymen, for seedling inoculation.

Guilan Province is the important habitat of alder in Iran. Frankia plays a significant role in nitrogen fixation and growth of alder trees. Unfortunately, the isolation, identification and application of Frankia-alder symbiosis have not been studied in Iran. Thus, this study is the first attempt to evaluate the effectiveness of Frankia coexists with endemic alder of Iran, to improve the growth and nutrition of the host plant. The aim of this study was to evaluate the potential of *A. Subcordata* to provide their N requirement through actinorhizal symbiosis and to determine the effects of inoculate source on nodulation, growth and N content of plants, Then we tested the symbiotic efficiency of 25 Frankia from crushed nodules of *A. Subcordata* of different sites in Guilan province.

MATERIALS AND METHODS

Collection of Frankia nodules

To prepare the Frankia inoculums, 25 different nodules of *A. Subcordata* roots were collected from Guilan province [12].The geographical position of each region was recorded with Global Positioning System (GPS). Fresh and active nodules (light color) from a single tree were harvested at each location. After collection, the nodules in a container with moist filter paper in the ice boxes were transferred to the laboratory [13].In the laboratory, soils adhering to the nodules washed thoroughly under running water. The nodules were surface sterilized with sodium Hypochlorite (1% active chlorine) and hydrogen peroxide (30%) for one minute, then rinsed several times with sterile distilled water [14].The nodules were stored in a refrigerator for about 3 weeks before they used for inoculation.

Preparation Frankia inoculums

About 1 g of each nodule Lobes was separated using sterile surgical blade, It was then crushed into Eppendorf tubes [15] contains a small amount of sterilized distilled water. Then, with 2% sucrose solution was brought to volume. Thus a total of 25 inoculums were prepared.

Seed collection and production of alder seedlings

Fruit cones were collected from an individual *A. Subcordata* tree in Talesh, western of Guilan. Seeds were harvested from the cones and stored in paper bags at room temperature. The seeds for ten minutes with a solution of sodium hypochlorite (1% active chlorine) were surface sterilized and then in the a Petri-dish containing moist filter paper exposed to light at room temperature until they germinated. The three germinated seeds were planted in a 4L plastic pots filled with sterilized sand and on a weekly basis until a week before the addition of the inoculums, were irrigated with the Hoagland nutrient solutions with low concentrations of nitrogen (one quarter) [16]. After three weeks, the seedlings were thinned and one seedling per pot was remained. Plants were raised in a glass-house at 25^{o}C and illuminated for 16 h/day. One week before inoculation, the plants were irrigated with1/4 Hoagland nutrient solution without N-content.

Experimental design

The experiment was conducted in a completely randomized design with three replications. Treatments include 25 inoculums along with a control. After the seedlings reached the age of six weeks, 10 ml of a well-fragmented inoculums suspension (approximately 30 mg fresh weight nodules) added to surrounding of root seedlings. Also Control seedlings received an equal volume of sterile suspension. Two weeks after inoculation and every week until harvesting, seedling were irrigated with1/4 Hoagland nutrient solution without nitrogen. During this period, growth in height and collar diameter was measured. Also After ten weeks, above and below ground parts of plants were harvested, weighed and dried at 70° C in an oven. Shoot, root and nodules dry weight, efficiency of nutrient uptake, the effect of microbial inoculation, nitrogen fixation and N content in the plant tissues were determined. Total N in plant tissues was determined by the Kjeldahl digestion method. Estimated mg N fixed per mg nodule dry weight was calculated according to NG [17]:

$$\frac{total\ N\ content\ of\ inoculated\ plant \quad N\ content\ of\ uninoculated\ plant}{total\ nodule\ dry\ weig\ \ t\ per\ plant}$$

The efficiency of nutrient uptake (ENuU), de ned as the amount of nutrients absorbed per unit root mass [18,19]was calculated as follows:

$$ENuU = \frac{Plant\ nutrient\ content\ (\mu g)}{Root\ biomass (mg)} \mu g\ mg^{-1}$$

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Microbial inoculation effect (MIE) was calculated [19]:

$$MIE = \frac{Dryweig\ \ t\ of\ inoculated\ seedling \quad Dry\ weig\ \ t\ of\ uninoculated\ seedling}{Dry\ weig\ \ t\ of\ inoculated\ seedling} \times 100$$

Statistical Analysis

Prior to analysis, the data were tested for normality and non-normally distributed data were square root transformed. All the data were subjected to statistical analysis using SAS software [26]. Where the F-test showed significant differences, Tukey test (P<0.05) used to determine the significance of mean differences between treatments.

RESULTS

The geographical position of the sites that we sampled root nodules was listed in Table 1.

Table1. The geoghraphical position of sampled root nodules of *A.subcordata*

Index	County	GPS		
AS34	Amlash	39 S	429736	4099363
AS40	Amlash	39 S	427202	4105429
AS41	Amlash	39 S	425871	4105628
AS50	Amlash	39 S	432543	4100144
AS45	Lahijan	39 S	403348	4108477
AS46	Lahijan	39 S	417984	4112383
AS47	Lahijan	39 S	418174	4109336
AS42	Langarod	39 S	419977	4105541
AS35	Roudsar	39 S	439863	4099164
AS48	Roudsar	39 S	439329	4096694
AS49	Roudsar	39 S	437622	4095347
AS36	Syahkal	39 S	399402	4104010
AS37	Syahkal	39 S	401136	4098352
AS38	Syahkal	39 S	401805	4099260
AS39	Syahkal	39 S	399401	4107877
AS43	Syahkal	39 S	402315	4101736
AS44	Syahkal	39 S	401951	4106472
AS29	Talesh	39 S	308973	4172642
AS30	Talesh	39 S	306929	4173078
AS31	Talesh	39 S	306154	4172001
AS33	Talesh	39 S	318067	4159443
AS51	Talesh	39 S	324347	4130264
AS52	Talesh	39 S	319390	4142930
AS53	Talesh	39 S	307064	4180190
AS32	Tonekabon	39 S	495955	4066777

Seedling growth characteristics

Analysis of variance showed that the use of Frankia inoculum had a significant effect on seedling growth characteristics (Table2). The means comparison revealed that the seedlings inoculated with Frankia AS29 and AS50 had the minimum and maximum height, respectively and significant increase of 83% was recorded(Table2).Overall, 50% of the inoculums increased the seedling height compared to control tratment. The mean comparison of diameters, showed that Frankia inoculums have increased the diameter of seedlings in 8 to 52% compared to control treatment (Table2).Minimum and maximum diameter of seedlings, with 2.5 and 5.2 mm, recorded in control and AS50 treatments, respectively. The mean comparison showed that dry shoot weight was increased from 5 to 182% in seedlings inoculated with Frankia crushed nodules than the control treatment. Minimum and maximum weight of shoots with 1.88 and 5.28g was observed in the treatment of AS50 and AS30, respectively. In comparison to the control, the shoot weight was reduced in treatment 36 (Table2). Mean comparisons showed that 84% of the Frankia inoculums enhanced root weight from14 to 163% than the control. Root weight increased about 163% in AS50treatment. However, the inoculums of 36 were reduced root weight of seedlings about 20% (Table2). Mean comparisons showed a significantly different between the weights of the root nodules. Nodules Weight varied between 0.047 to 0.2 g per plant. Minimum and maximum weight of nodules was observed in treatment AS29 and AS50 respectively (Table2).

Nitrogen Concentration and Efficiency of Nitrogen uptake

The ANOVA indicated that Frankia inoculums significantly influenced the nitrogen concentration and efficiency of nitrogen uptake of *A. Subcordata* seedlings, as compared to non-inoculated plants ($p<0.01$) (Table2). Mean comparisons showed that control seedlings have the minimum concentration of nitrogen than Frankia-inoculated seedlings. Frankia inoculums could increase the nitrogen concentration of seedlings from 11.11 to 130.2%.The lowest and the highest concentrations of nitrogen was observed when *A. Subcordata* seedlings inoculated with control and AS50 respectively (Table2). Efficiency of nitrogen uptake was higher for all Frankia-inoculated seedlings (Table2). The efficiency of nitrogen uptake of Frankia-inoculated seedlings were10.8–154.5% higher compared to control. Efficiency of nitrogen uptake had a positive correlation with nodule weight, N content, and seedling biomass and growth (Table3).

Biomass of Nodules

The roots of the un-inoculated plants did not have nodules. A significant differences detected in nodules weight per plants among all treatments. All seedlings that inoculated with Frankia had nodules (Table2). The nodule dry weights varied from 0.047–0.23g in inoculated treatments Seedlings inoculated with AS50 and AS48 showed the highest nodule weight compared to other treatments (Table2).

Effect of Microbial Inoculation

There was a significant difference in MIE of inoculated seedlings (Table2). Seedlings inoculated with Frankia had MIE between 1.78 to 63.25%, except seedlings inoculated with AS36 crushed nodules that has a negative MIE (-3.3%)(Table2).

Table 2.Mean comparison of effects of different Frankia crushed nodules on growth characteristics of *A. Subcordata* seedlings

Site	MIE %	ENuU μg/mg root	N Content %	Dry weight (g)			Diameter (mm)	Height (cm)
				Nodule	Root	Shoot	Shoot	Shoot
1	0	56.36	1.89	0	0.63	1.87	2.49	26.33
29	3.95	76.02	2.36	0.047	0.63	1.97	2.99	19.33
30	4.08	70.85	2.72	0.07	0.73	1.88	3.24	21
31	1.78	87.43	2.7	0.077	0.61	1.93	3.16	22.33
32	15.95	62.45	2.12	0.11	0.79	2.19	3.93	24
33	12.42	65.51	2.19	0.093	0.72	2.14	3.89	23
34	14.36	91.25	2.99	0.11	0.75	2.17	3.83	24.67
35	2.55	72.16	2.66	0.097	0.69	1.87	3.44	24.33
36	-3.33	80.55	2.99	0.1	0.51	1.36	3.14	25.33
37	24.18	89.82	3.25	0.14	0.88	2.43	3.86	28
38	24.99	81.71	2.96	0.14	0.91	2.53	3.89	28
39	25.77	89.43	3.08	0.11	0.87	2.54	4.27	23.67
40	38.68	91.03	3.65	0.16	1.26	2.92	4.34	29.33
41	36.78	85.73	2.9	0.15	1.05	3.08	4.27	28.33
42	34.68	91.96	2.8	0.14	0.91	2.95	3.92	28.67
43	4.38	85.21	2.53	0.11	0.61	2	2.69	26.67
44	35.48	81.33	2.78	0.16	0.99	2.89	4.03	30.33
45	40.24	88.29	2.95	0.12	1.07	3.18	4.26	25
46	29.91	82.7	2.92	0.13	0.96	2.7	4.24	26
47	22.05	75.08	2.72	0.1	0.85	2.36	3.94	23.33
48	45.51	89.94	3.08	0.19	1.19	3.46	4.21	33
49	53.54	92.07	3.31	0.18	1.5	4.18	4.45	31.67
50	63.25	143.46	4.35	0.23	1.66	5.28	5.22	35.33
51	37.65	123.59	2.94	0.13	0.88	3.2	3.83	27.33
52	43.22	96.66	3.31	0.18	1.13	3.28	4.17	32
53	47.22	81.96	2.97	0.18	1.26	3.49	4.32	31.67
Pr> F	<0.0001	0.0018	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
C.V%	33.99	21.7	12.96	19.59	23.44	14.69	9.85	10.83
LSD	25.38	57.83	1.16	0.07	0.68	1.25	1.18	9.25

N_2 –fixation

The capacity of N_2-fixation was estimated by calculating the mg N in shoots per mg of nodule dry weight [17]. Frankia inoculums had a significant effect on N_2-fixation by *A.subcordata* seedlings (Table2).The

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amount of nitrogen fixation ranged between 0.08 to 1.13 mg/mg nodule. The highest amount of xed nitrogen was recorded in seedlings inoculated with AS50 (1.13mg N/mg nodule). However, Inoculation with AS36, had little effect on the amount of xed nitrogen compared to control (Fig.1).There was significant linear correlation between N_2-fixation and other characteristics of seedlings.MIE and height of seedlings respectively, have the highest and lowest correlation coefficient with nitrogen fixation (Table3).

Table.3. Pearson's correlation coefficient between N_2-fixation and seedling characteristics

	Height	Dia	Shoot	Root	N	Nodule	MIE	ENU
Diameter	0.419**							
Shoot D.W	0.644**	0.762**						
Root D.W	0.676**	0.800**	0.890**					
N	0.522**	0.594**	0.587**	0.589**				
Nodule D.W	0.550**	0.642**	0.642**	0.650**	0.539**			
MIE	0.507**	0.780**	0.875**	0.812**	0.477**	0.759**		
ENU	0.246*	0.259*	0.496**	0.153	0.617**	0.303**	0.362**	
N_2-fixation	0.270*	0.726**	0.780**	0.711**	0.728**	0.698**	0.817**	0.540**

*. Correlation is significant at the 0.05 level **. Correlation is significant at the 0.01 level

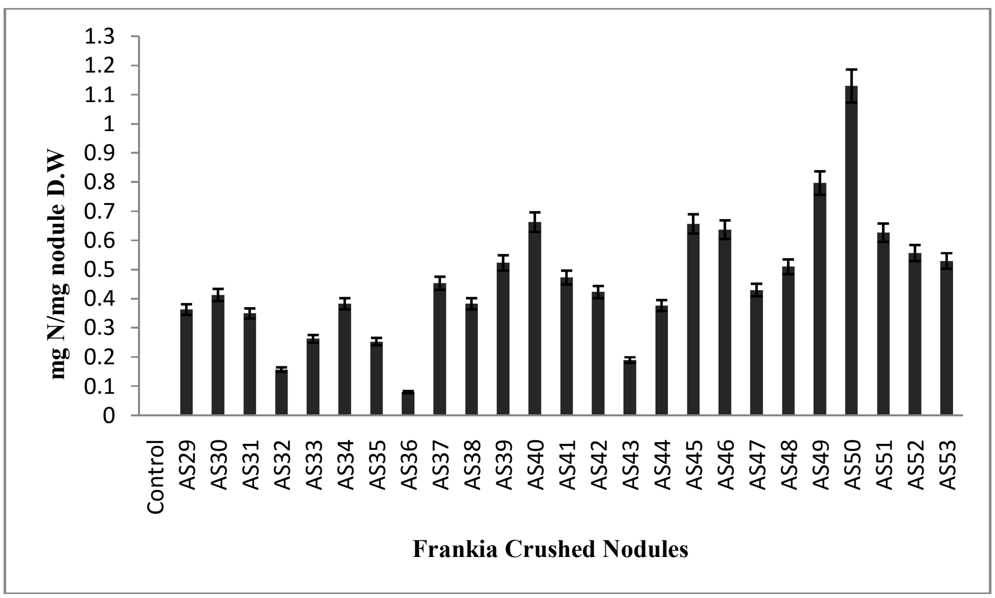

Fig. 1. Mean comparison of effects of different Frankia crushed nodules on N_2-fixation of *A. Subcordata* seedlings (LSD=0.46)

DISCUSSION

The present results demonstrated that the growth and N_2-fixation of *A. subcordata* seedlings signi cantly increased after inoculation with Frankia compared to un-inoculated seedlings. Inoculation of Frankia crushed nodule suspensions improved diameter and shoot dry weight of *A.subcordata* seedlings by 45% and 57%, compared to control. Rose and Youngberg [20] finding the same result that Frankia enhance the growth, nodulation, and N_2- xing capacity of actinorhizal plants. Also Yamanaka *et al*. [21]) reported that the growth and nodulation of *A. tenuifolia* signi cantly increased after inoculation with Frankia. The increased growth characteristics of inoculated seedlings could be the result of increased efficiency of nutrient uptake, inoculation efficiency and nutrient availability. The existence positive correlations between studied characteristics supported this idea.

In our study, nitrogen composition of *A. subcordata* affected by Frankia inoculation. The increased content of N in seedlings inoculated with Frankia crushed nodules indicated their e ectiveness in improving the nutrition of *A. subcordata*. N_2- xation by the Frankia may play a key role for inoculated alders. The increased nutrient content of inoculated seedlings may be advantageous for these seedlings during out-planting as nutrient status of the seedlings in uence resistance to various stresses [22].

Nodule dry weights that formed in the root of inoculated seedlings positively correlated with N_2- xation, growth and seedling biomass. Wheeler *et al.* [23] reported a similar response of increasing the nodule mass of *Alnus* to inoculation with crushed nodule suspension. This result is in agreement with the finding [24, 25]. Rojas *et al*. [25, 27] reported a positive correlation between seedling and nodule biomass in red alder (*Alnus rubra*). According to Arnone and Gordon [28] this correlations con rmed that symbiotic N_2- xation is dependent on host plant photosynthesis. Also Sellstedt *et al*. [29] reported that spore negative nodule homogenate produced higher biomass than spore positive nodule homogenate in *A. incana*.

CONCLUSIONS

Our results confirmed the importance of using Frankia crushed nodules in seedlings of alder. The Frankia AS50 has the highest effectiveness for growth and nitrogen fixation in *A. subcordata* than others. The different results in the efficiency of our tested inoculums indicate that superior Frankia crushed nodules may be discovered and developed. Thus selection sources of inoculums that have a considerable influence to *A.subcordata* and optimizing sustainable production of these inoculums is needed.

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