



Original article

Biological role of mycorrhizal fungi on the assimilation and transportation of carbon and nitrogen to *Anacamptis palustris* and *Anacamptis laxiflor*

Sameera A. Alghamdi*

Department of Biological Sciences, Faculty of Science, King Abdulaziz University, P.O. Box 8020, Jeddah 21589, Jeddah, Saudi Arabia

Princess Dr. Najla Bint Saud Al-Saud Center for Excellence Research in Biotechnology, King Abdulaziz University, P.O. Box 8020, Jeddah 21589, Jeddah, Saudi Arabia



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ABSTRACT

Fungal is a physiological trail and its understanding in the assimilation with the transfer of carbon (C) cum nitrogen (N) or (C/N) to orchid-seedlings have not been determined. Labelled stable isotopes ^{13}C and ^{15}N were used to plan the flow of C and N between orchid plants and mycorrhizal connotations in-terms of bulk transfer for C/N. This study attends to comprehend the mechanism, supporting mycorrhizal fungi which influences on orchid-seedling growth. Determined integration and transfer of C/N from amino acids (AA), ammonium nitrate (NH_4NO_3) and sugar for orchid-plant may lead to understand these mechanisms. This current study tries to estimate the importance of organic compounds as a source for C/N over the inorganic- NH_4NO_3 . Generally, after begging of germination and when it is found to be associated to the nutrient resource, organic compound enhance the biomass accumulation of two orchid species. AA significantly increase the mass of ^{13}C assimilated by two species. With amino acids the concentration of ^{13}C in two species was greater than with NH_4NO_3 and sugar. At another phase, amount of ^{15}N content shoots was a higher value in *Anacamptis laxiflora* shoots assimilated substantially additional of ^{15}N with NH_4NO_3 plus sugar compared with ammonium nitrate only. This study showed that two terrestrial orchids species are reliant on organic compounds as a source of carbon and nitrogen more than inorganic compounds.

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1. Introduction

Culturing terrestrial orchids species from seed is a big challenged where orchids species require a symbiotic fungal partner to germinate productively. They consist of undifferentiated tissues and lacking cotyledon or endosperm. Orchid-plant generates enormous small-seeds, have inadequate amount of carbon (C) and essential elements. The orchid reliant on fungal spouse for supplying of C and essential-nutrients required for growth and development to the adult plant (Rasmussen, 1995; Arditti and Ghani, 2000; Dutra et al., 2008; Fay, 2018). Although the orchid depends

on the mycorrhizal fungi to provide the necessary elements, orchid will create green sprouts, perhaps improves the mycorrhizal fungi, through the mutualistic symbiosis with replying carbon exploit in an initial life-cycle (Cameron et al., 2006, 2008). The fully mycoheterotrophic orchid and the initially mycoheterotrophic-orchid-species require their partner (mycorrhizal) for carbon provide through their life stage (Johnson and Kane, 2007; Smith and Read, 2008; Merckx and Freudenstein, 2010; McCormick et al., 2018).

Terrestrial orchids species, which constitute about a third of orchid species, they are severe danger for extinction, because of a variety of hazarding processes. In reaction to menaces to orchid species, incorporated multidisciplinary maintenance approaches, including *in vitro* methods, are assumed to understand the basic biology of the symbiosis and to assist the protection of rarity species.

Anacamptis palustris is extensively distributed in the western Mediterranean region. It is found in Algeria, Greece, Tunisia and Saudi Arabia (Cozzolino et al., 2003; Bateman et al., 2003; Swartz and Dixon, 2009; Seaton et al., 2010). *Anacamptis laxiflora* species

* Address: Department of Biological Sciences, Faculty of Science, King Abdulaziz University, P.O. Box 8020, Jeddah 21589, Jeddah, Saudi Arabia.

E-mail address: saalghamdi17@gmail.com

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distributed in several areas in Mediterranean basin. It is growth in southern Europe, Turkey and Greece (Arduino et al., 1996; Sgarbi et al., 2007). Some current researches show's that, studying both environmental factors and biological aspects are required to understand highly specific mycorrhizal to explain rarity in Mediterranean terrestrial orchids (Arduino et al., 1996; Bateman et al., 2003; Swartz and Dixon, 2009; Wraith and Pickering, 2019).

The dependence on mycorrhizal fungi to get essential elements in the adult's phase is slightly acknowledged. Although, by measured natural abundance isotopic enrichment in some orchid species have found high augmentation of tissues in ^{13}C and ^{15}N that grand evidence of carbon and nitrogen being co-transferred from fungal partner to the plant (Arditti, 1992; Leake, 1994; Gebauer and Meyer, 2003; Cameron et al., 2006, 2008; Leake and Cameron, 2010, 2012). Irradiance in terrestrial gives the ability to understand the basis of mechanism for the differences in orchid seedling development. Therefore, I studied the assimilation and transfer of isotopically labelled ^{13}C and ^{15}N to solution the mechanisms of carbon and nitrogen transfer from fungal partner to plant. Therefore, the present study aimed to understand the capacity of fungal mycelia to assimilate and transfer of amino acids (AA); an organic sources/ammonium nitrate (NH_4NO_3); an inorganic source, with/without glucose to *A. palustris* and *A. laxiflora*. Small quantities of organic nutrients can be increased ^{13}C and ^{15}N augmentation of orchid-tissues. The accumulation in orchid-plant tissues will determine a partiality for an organic compound's sources of carbon/nitrogen more than inorganic compounds.

2. Materials and methods

2.1. Collection of plants

The Mediterranean terrestrial orchid seeds of *A. palustris* and *A. laxiflora* were collected from Kew's Millennium Seed Bank (MSB) in West Sussex. Cultures of fungal partner for *A. palustris* and *A. laxiflora* species have grown through intracellular hyphal pellets through orchid-root of surface sterilization moved towards petri-dishes which includes the non-nutrient agar, in the plant agar, a fresh culture have been created which were then utilized for the fertilization of the experimental microcosms.

2.2. Experimental microcosms

Thirty Petri dishes were set up for every terrestrial species. Petri dishes were arranged having the 50 mL of Rorisons nutrient-agar. The plant agar concentration was 12g L^{-1} and 5.5 was the pH media autoclaving for 45 min at $121\text{ }^\circ\text{C}$. The inoculation was done through the petri dishes in the middle of the 5 mm disc in mycorrhizal fungi. Approximately 60 seeds for each terrestrial species has sterilised using 4% of calcium-hypochlorite which includes Towline 80 (2 drops; Sigma-Aldrich, UK) and then using the rotary-shaker for 10 mins; moved towards the ultra-violet sterilized laminar air-flow for all the dishes (HWS Series - AS1386). By used Whatman N^0 1 filter paper seeds were filtered (Sigma-Aldrich) and washed through 100 mL of sterilized water. Prior incubated at a constant $18\text{ }^\circ\text{C}$ the Petri dishes were covered in Parafilm.

2.3. Experimental design

Seedling were left to cultivate for two months with their mycorrhizal fungi. The complete similar-size seedlings were supplied with identical substance involved in this study. Thirty dishes were arranged for each terrestrial-species in 3 compartment-plates of 9-cm in diameter. The initial compartment involves 12 g L^{-1} mixture

of plant-agar for the one-fourth strength in the Rorisons' nutrient solution by carbon provided as stable isotopes labelled ^{13}C and nitrogen-sources as provided with the stable isotopes categorized as ^{15}N AA combination appeared as-an organic source or (stable isotopes defines as $^{15}\text{N-NH}_4\text{NO}_3$) as an inorganic sources, combined with/without labelled ^{13}C glucose have been added for degerming the $^{13}\text{C}/^{15}\text{N}$ enhancement through invitro. Labelling has the similar quantity for $^{15}\text{N}/^{13}\text{C}$, irrespective for chemical-sources form through the addition of stable isotopes were shown in Table 1. The mixture of AA was strained and bleached with $0.2\text{ }\mu\text{M}$ in addition of the pore-membrane to agar which was autoclaved, while NH_4NO_3 were filtered/sterilized in the autoclave with an agar. However, pH media were previously regulated for 5.5 for 45 mins at $121\text{ }^\circ\text{C}$ for autoclaving.

2.4. Seedling growth rates

Using the plant agar, seedlings were developed as per the required concentration for contact with carbon/nitrogen compounds in the second-subsequent compartment. The final compartment comprises plant agar with couple of sides to deliver the hydrophobic-block for avoiding the elements from the involvement of 2nd compartment which includes the C/N sources in the plant seedlings to prevent the contact of fungal hyphae (Table 1).

Using the parafilm, dishes were covered directly to evade the infection. The aluminium foils were used for 30 dishes for protection in the dim-shadow through polythene bags (plastic covers); placed in skilful environment plant growing chamber for a couple of months at $18\text{ }^\circ\text{C}$ through extended fungal hyphae reaches to inhabit the plate. There were ten replicate dishes for each treatment. Each fresh terrestrial plant weight was measured and plant sample were placed in 1.5 mL of an DNA tube and then placed for 3-days in the freezer. Later on, lid of the tubes was opened and draped with parafilm and punctured in prior cryodesiccation to make the substance more suitable for transport of a minimum of 6 days. All the dried terrestrial plant samples were measured and placed in the tin cups. Through the Isotope Ratio Mass Spectrometry (IRMS), an element isotopes ratio was screened in terrestrial orchid-plant tissues.

2.5. Statistical analysis

The variations in means of biomass and isotope enrichment were determined using 2way ANOVA analysis through the Turkey's multiple comparison tests. All comparison set has the same variance. A p values lower than 0.05 is considered as significant.

3. Results

The plant *A. palustris* is the shoots for biomass connected with Carbon and Nitrogen, tagged isotopically through the fungal partners, substantially more than the served through the labelled sources. This sort of Nitrogen sources NH_4NO_3 (with glucose or without glucose) or amino acids has not affected (Fig. 1; Table 2). The shoots for biomass of *A. laxiflora* plants were disengaged through the labelled sources was substantially different from the

Table 1
Carbon and nitrogen Sources ^{13}C , ^{15}N extents in the experiment.

Carbon and nitrogen sources	Isotopes	Labelled (mg L^{-1})
Amino acids	^{15}N	39.56
	^{13}C	
Ammonium nitrate + sugar	^{15}N	13.17
	^{13}C	48.23
Ammonium nitrate only	^{15}N	13.17

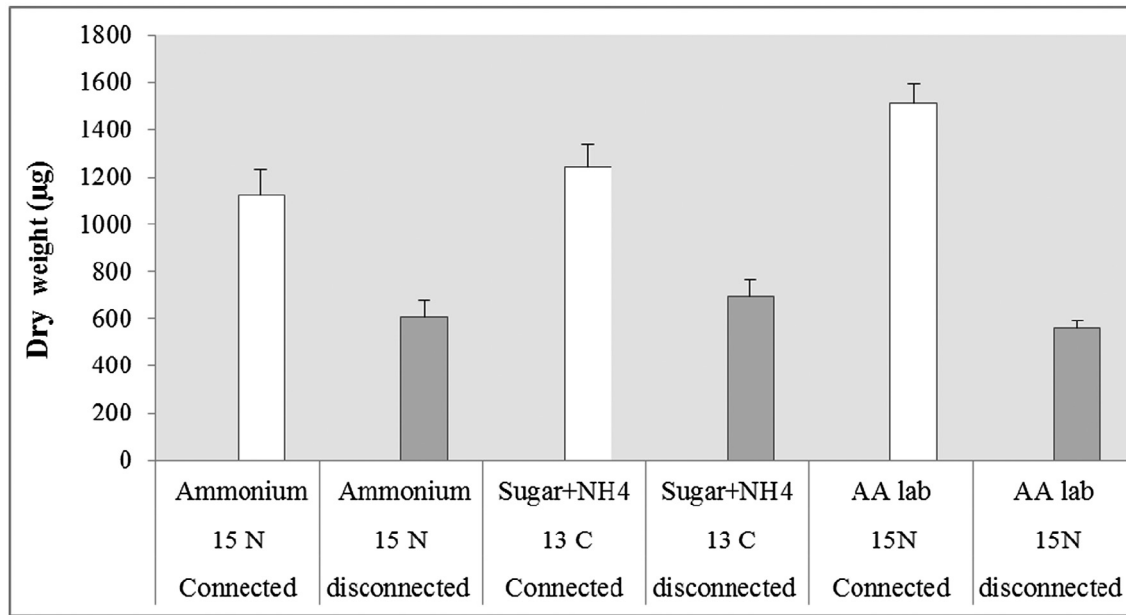


Fig. 1. Biomass of *A. palustris* shoots served and connected through the isotopically labelled Carbon-Nitrogen sources. The variations between mean dry weight were performed with 2-Way ANOVA analysis followed by Tukey's multiple comparison test (TMCT) ($p < 0.05$; Table 2). $p < 0.05$.

Table 2
Shoot biomass of two orchid species by used 2-way ANOVA.

Species	Factor	d.f.	F	P
<i>A. palustris</i>	Connection	1,30	40.35	0.001
	Substrate	2,30	7.46	0.064
	Connection × Substrate	2,30	6.15	0.770
<i>A. laxiflora</i>	Connection	1,30	50.66	0.001
	Substrate	2,30	1.77	0.012
	Connection × Substrate	2,30	4.21	0.001

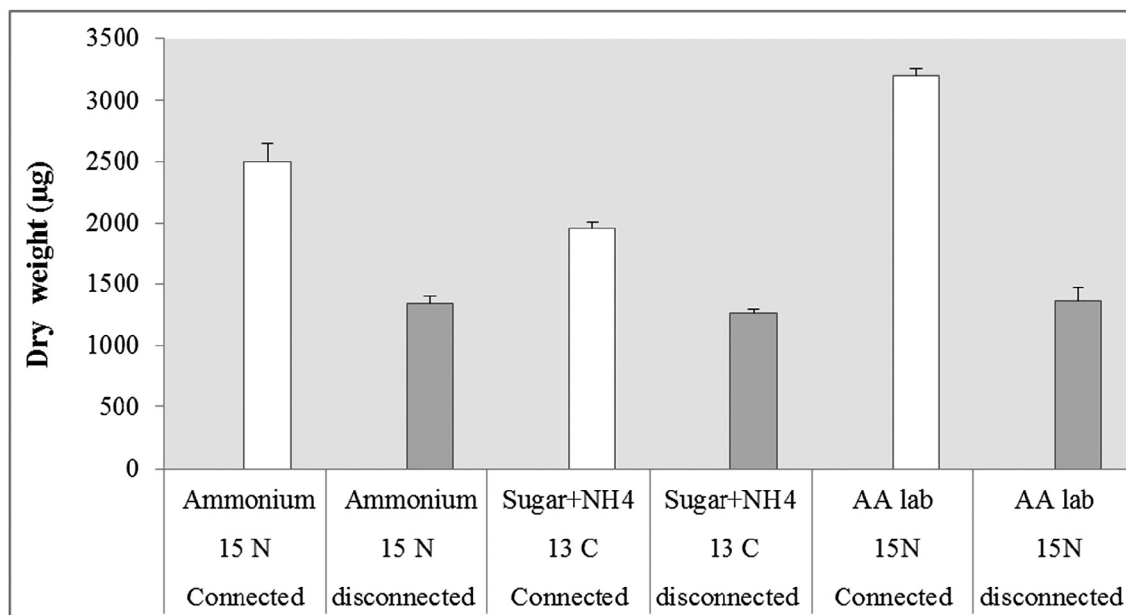


Fig. 2. Biomass of *A. laxiflora* plant shoots were linked via their fungal partners by the isotopically labelled Carbon-Nitrogen sources. In each bar 2-way ANOVA analysis was performed to differentiate between the mean dry-weight followed by TMCT (Table 2). $p < 0.05$.

connected and isotopically labelled carbon and nitrogen sources through their fungal partners. However, the shoots of biomass were found to be greatly associated with amino acid treatment in plant growth with ammonium/ ammonium and glucose (Fig. 2; Table 2).

The tissue or mass concentration of *A. palustris* shoots did not take up significant quantities of ^{13}C once served through the tagged sources of tiny ^{13}C enhancement was observed in the plants provided through ^{15}N -labelled ammonium and sugar 4.57 μg . The total ^{13}C were supplied to the shoots through mycorrhizal fungi getting into amino acids were 38.17 μg , whereas, the treatment with NH_4NO_3 and glucose were 29.88 μg (Fig. 3; Table 3). However, a significant mass of ^{13}C were documented in *A. palustris*, plant-shoots supplied by ^{13}C -labelled amino acids when compared with the provided ^{13}C -labelled glucose and NH_4NO_3 (Fig. 3; Table 3). The isotopes concentration in shoot-tissues were detected separately in a relationship with non-statistically significant differences in the ^{13}C -content of shoots with an irrespective of whether the seedling was provided with ^{13}C -labelled NH_4NO_3 with or without glucose (Fig. 4; Table 4). The juvenile *A. laxiflora* shoots were not has the significant amount of ^{13}C when disconnected through the tagged sources. The complete ^{13}C received for the shoots of fungal partners containing the amino-acids were 20.70 μg (Fig. 5). However, a positive greater mass was predicted with *A. laxiflora* shoots delivered through ^{13}C -labelled amino-acids when equated with the provided through NH_4NO_3 and sugar was 18.58 μg (Fig. 5; Table 3). The isotope concentration of *A. laxiflora* shoot-tissues showed the positive difference with ^{13}C of shoot contents (Fig. 6; Table 4). The entire ^{13}C content of *A. laxiflora* shoots were higher with amino-acids. Apart from these, *A. laxiflora* plants provided by NH_4NO_3 with glucose is assimilated highly with ^{13}C than the plants excluding the glucose. Generally, the complete ^{15}N detected in *A. palustris* shoots were not associated and influenced through Nitrogen sources (Fig. 7; Table 5). Complete ^{15}N content of *A. palustris* shoots was the highest values provided with plants by

^{15}N -labelled amino-acids (Fig. 7; Table 5). However, *A. palustris* plants provided through glucose has not assimilated significantly further ^{15}N through labelled- NH_4NO_3 only (Fig. 7; Table 5). ^{15}N concentration of *A. palustris* shoots has assimilated more strongly in ^{15}N with amino-acids measured from NH_4NO_3 . Moreover, there was a significant growth in the ^{15}N assimilation in the plants provided through glucose and NH_4NO_3 (Fig. 8; Table 6). In the *A. laxiflora* plant shoot with the mass of ^{15}N has assimilated consistently with ^{15}N from the NH_4NO_3 and inclusion of sugar when equated with amino-acids (Fig. 9; Table 5). The transferred volume of ^{15}N in the *A. laxiflora* shoots with the fungal partners obtained the ammonium was 5.88 μg ; consistently more extensive than by the transfer of glucose 4.67 μg (Fig. 9; Table 5). A higher greater concentration of ^{15}N was perceived in the *A. laxiflora* plant shoots, provided through NH_4NO_3 and inclusion of sugar through amino-acids. Furthermore, *A. laxiflora* plants provided through glucose has not assimilated significantly further ^{15}N from the labelled NH_4NO_3 than the plants with no glucose (Fig. 10; Table 6).

4. Discussion

Many Mediterranean terrestrial orchids are presently at excessive risk for extinction as an effect of threatening behaviours. Therefore, in this present research was focussed on two different species or orchid in Mediterranean area to understand the influence of the mycorrhizal fungi on development and juvenile nourishment in orchid plants. In two terrestrial orchid species; *A. palustris* and *A. laxiflora* after germination has been established previously. Then only converts the photosynthetic based on the shoot's creation (Stewart and Kane 2010; Martos et al, 2012; Pellegrino et al., 2014; Swarts and Dixon 2017; Li et al., 2018). Moreover, separation of fungal partners to access the nutrient supplies will directed by the significant decrease in the development of the total species compared with the plants and its associated

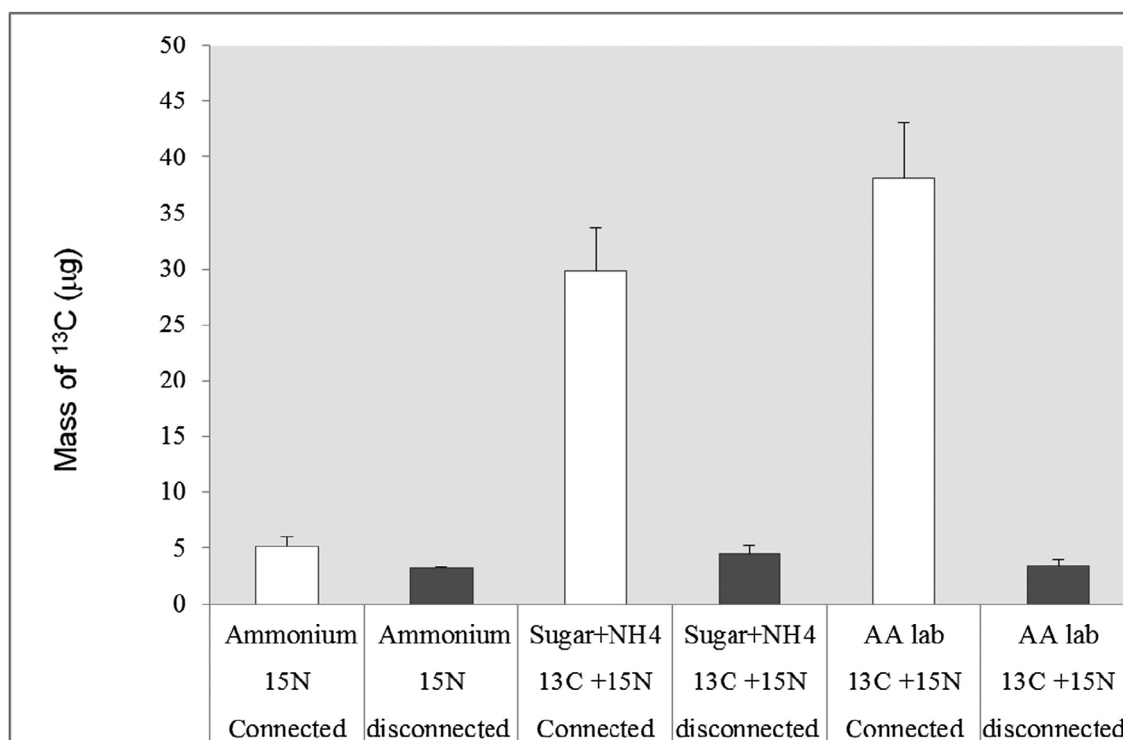
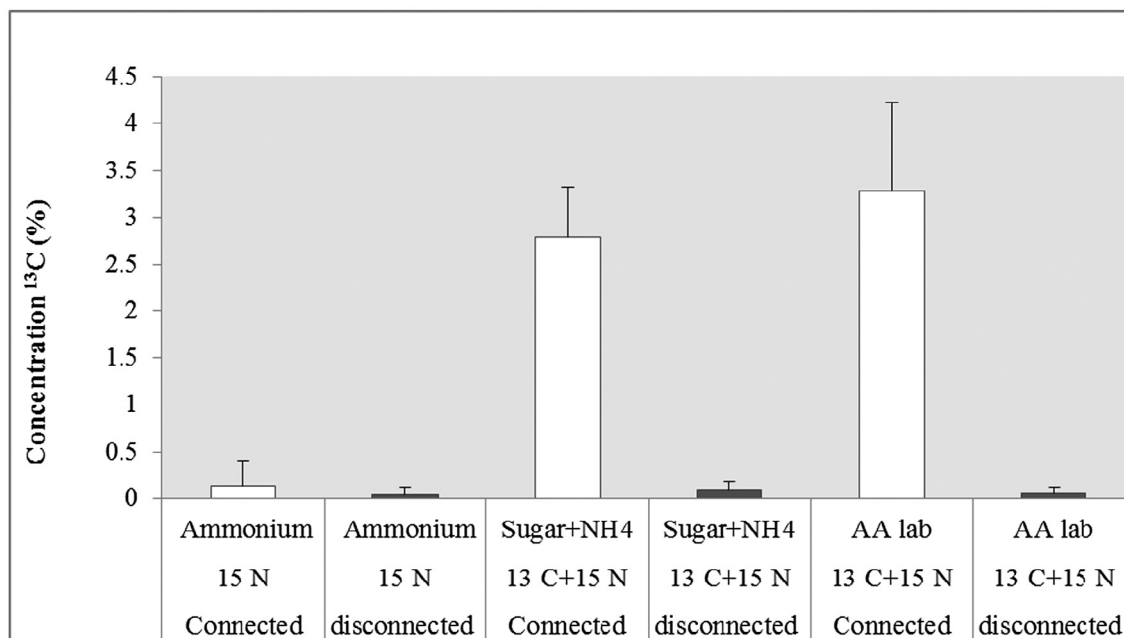


Fig. 3. ^{13}C mass in *A. palustris* shoots after supplying ^{15}N - ^{13}C labelled organic/inorganic compounds through the isotope labelled Carbon-Nitrogen compounds. The 2-way ANOVA analysis was used in each bar to measure the alterations between the mean mass followed by TMCT (Table 3), $p < 0.05$.

Table 3
Shoot mass of ^{13}C of two orchid species by used 2-way ANOVA.

Species	Factor	d.f.	F	P
<i>A. palustris</i>	Connection	1,30	87.46	0.001
	Substrate	2,30	52.20	0.001
	Connection \times Substrate	2,30	36.22	0.001
<i>A. laxiflora</i>	Connection	1,30	96.44	0.001
	Substrate	2,30	68.53	0.003
	Connection \times Substrate	2,30	60.21	0.001

**Fig. 4.** ^{13}C concentrated shoots in *A. palustris* of connected through isotopically labelled C-N sources. The Two-way Anova analysis was implemented between each graph and differences between the mean concentrations (Table 4). $p < 0.05$.**Table 4**
Shoot concentration of ^{13}C of two orchid species by used 2-way ANOVA.

Species	Factor	d.f.	F	P
<i>A. palustris</i>	Connection	1,30	98.77	0.001
	Substrate	2,30	40.35	0.088
	Connection \times Substrate	2,30	30.45	0.661
<i>A. laxiflora</i>	Connection	1,30	113.08	0.001
	Substrate	2,30	60.12	0.003
	Connection \times Substrate	2,30	60.35	0.001

resources, evidently representing the robust dependence at juvenile orchid-plants towards their fungal partners for the progress. Based on this issue, it has remained as the environmental resources delivers through the fungal partner at the initial phase of the growth i.e., juvenile orchid-plants are easily gaining nutrition's through the mycorrhizal fungi which remains incompletely heterotrophic, obtaining the significant carbon support. The reliance of orchid plants on fungal partner possibly, significantly varies through species-species particularly in the initial phase of the growth as terrestrial orchid plants remains mycorrhizal during their life-cycle (McCormick et al., 2018; Zhang et al., 2018). Some studies showed in the adult orchid plants as fungal partner provide essential nutrients, particularly Nitrogen and Phosphorous in restoration of Carbon. This present study is established as Carbon-Nitrogen gained from amino-acids, which was assimilated through mycorrhizal fungus of orchid-plants are shifted to the

seedlings of Orchid; probably due to the proper sustenance from heterotrophic evolution.

Martin and Botton (1993) conducted a study on Ectomycorrhizal fungi and concludes as fungi transports the amino-acids into their specific hosts plant and it delivers an establishment pathway of fungus to plant (carbon-nitrogen) in the mycorrhizal symbiosis. Still the query exists as in which position does the carbon transports from plants to the fungus. Smith (1967) proposes as carbon flux through the mycorrhizal fungi towards the orchid-plant which occurs through the fungal sugar trehalose. Moreover, Cameron et al. (2008) proposed as amino-acid could be the candidate for the transfer of molecule and confirms through the applying co-labelling method with ^{13}C - ^{15}N as labelled substrates.

An extreme rise in ^{13}C mass were transferred by the protocorm was realised in *A. palustris* with an existence of an amino-acids. Thus, it can be recommended as orchid-plant is a reliant on an

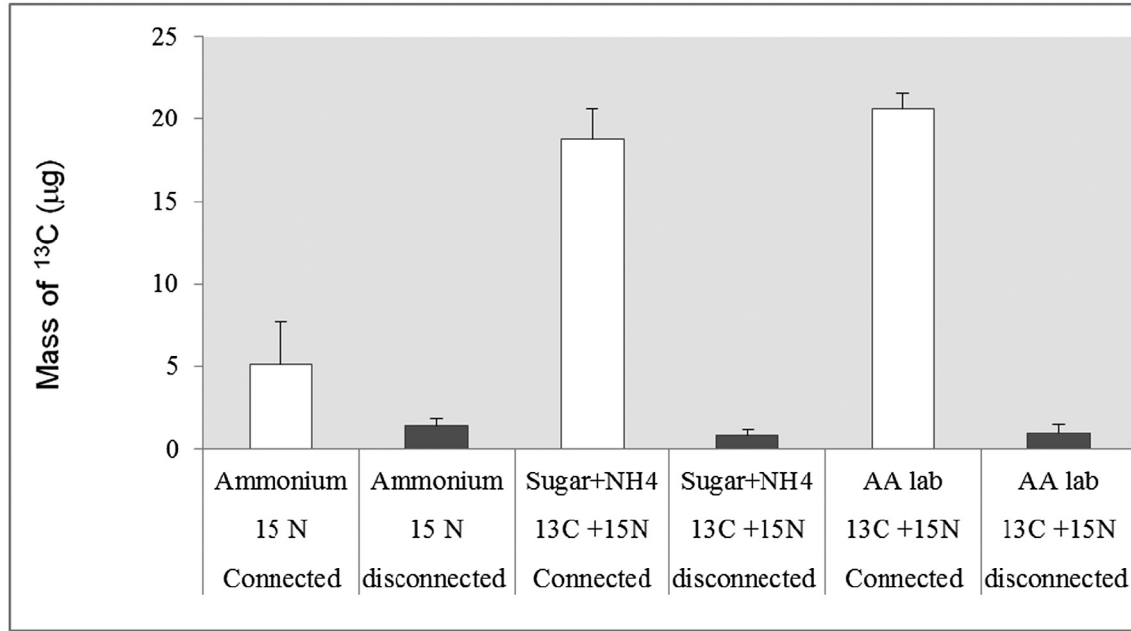


Fig. 5. Mass of ^{13}C in *A. laxiflora* plant shoots connected through isotopically labelled C-N sources through their fungal partners using ANOVA analysis (two-way) and TMCT (Table 3). $p < 0.05$.

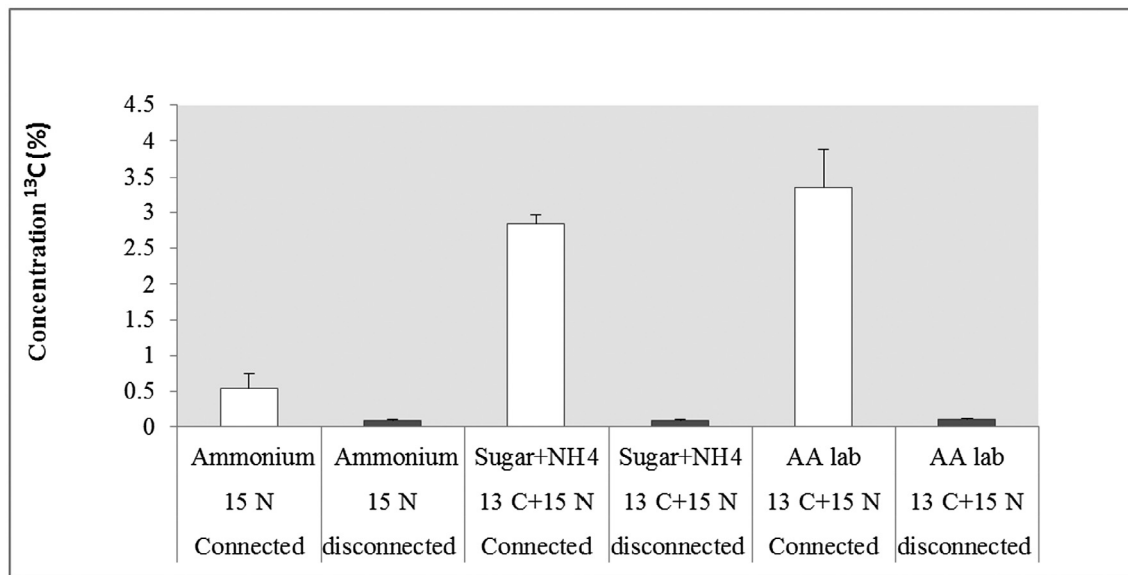


Fig. 6. Concentration of ^{13}C shoots of *A. laxiflora* connected isotopically via Carbon-Nitrogen sources through the fungal partners. In every graph, 2-way ANOVA analysis was used to differentiate between the mean concentration of ^{13}C (Table 4). $p < 0.05$.

organic material (ex; amino-acids), assimilated through mycorrhizal fungi. Both the carbon and nitrogen were transferred into the orchid-plant. High concentration of ^{13}C enrichment in protocorms provide through the amino-acid as a source as divergent to the glucose, Nitrogen evidences of mineral suggests that amino-acid could be fundamentally used as a carbon source through fungus and which can take care of complete transportation (Gebauer and Meyer 2003; Cameron et al. 2006). Moreover, positive association between ^{13}C or ^{15}N can uptake the bio-mass, realized in the maximum species proposed source-sink relation leads to this process, growth in the orchid-plant which leads to the resource assimilate.

Based on the ^{15}N isotope tracers, the current study shows two terrestrial-species obtained through substantially quantities of Nitrogen by the fungal partner. A strong proof of orchid-species underlies in this study is mainly reliant upon the mycorrhizal fungi in the nitrogen resources. The orchid-plant have an extensively deficient root-system and further upon they adopt the nutrition assimilation straight through the soil as per the rough nature, bound size and demonstrating the mycorrhizal fungi as the main track of the nutrient moves in the orchid-plant from the soil (Leake, 1994). High quality of ^{15}N in an *A. palustris* provides within the amino-acid sources showed as amino-acids are used as Nitrogen sources through the fungus. Earlier study from Majerowicz

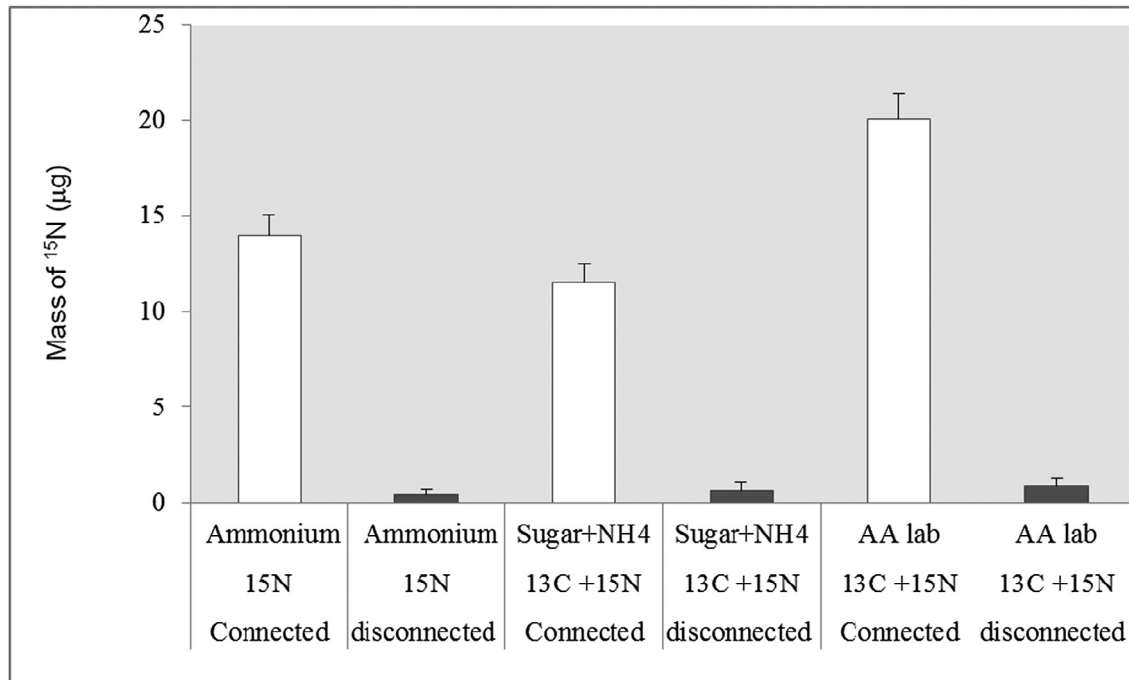


Fig. 7. Mass of ¹⁵N shoots of *A. palustris* connected by isotopically labelled Carbon-Nitrogen sources through their fungal infections using 2-way ANOVA analysis followed by TMCT for each graph and differences between mean mass (Table 5). $p < 0.05$.

Table 5
Shoot mass of ¹⁵N of two orchid species by used 2-way ANOVA.

Species	Factor	d.f.	F	P
<i>A. palustris</i>	Connection	1,30	130.75	0.001
	Substrate	2,30	18.80	0.062
	Connection × Substrate	2,30	13.45	0.081
<i>A. laxiflora</i>	Connection	1,30	88.40	0.001
	Substrate	2,30	6.80	0.041
	Connection × Substrate	2,30	5.35	0.001

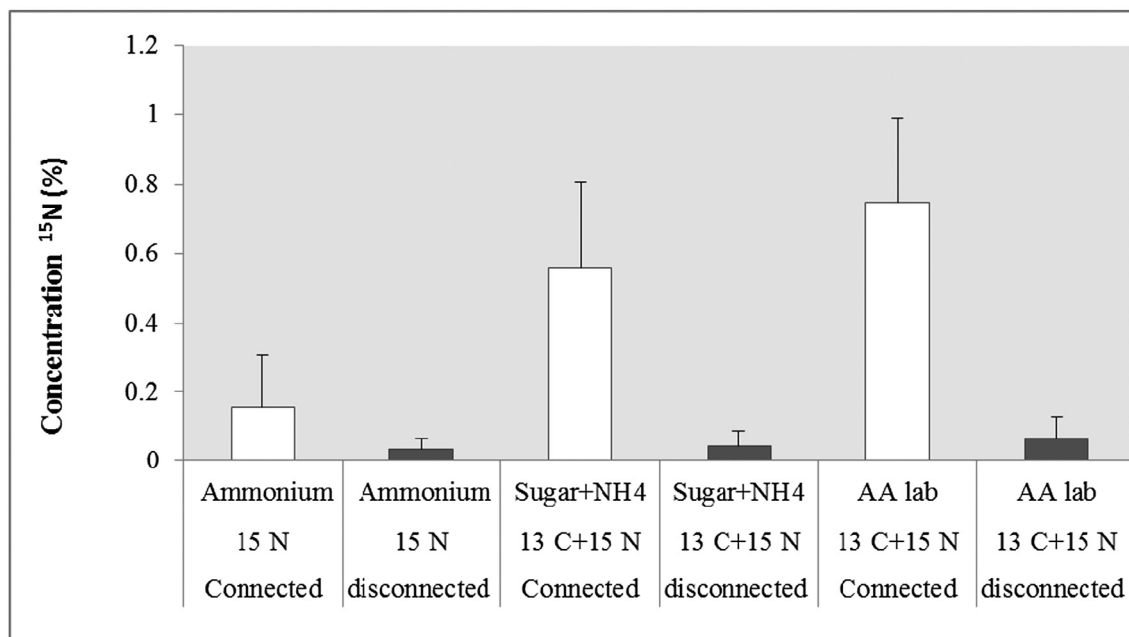


Fig. 8. ¹⁵N concentration in the *A. palustris* shoots connected from isotopically labelled Carbon-Nitrogen sources. In each and every graph, the differences between mean concentration were solved using 2-way ANOVA analysis and TMCT (Table 6). $p < 0.05$.

Table 6
Shoot concentration of ^{15}N of two orchid species by used 2-way ANOVA.

Species	Factor	d.f.	F	P
<i>A. palustris</i>	Connection	1,30	112.14	0.001
	Substrate	2,30	15.25	0.015
	Connection \times Substrate	2,30	18.7 5	0.001
<i>A. . laxiflora</i>	Connection	1,30	152.57	0.001
	Substrate	2,30	2.85	0.078
	Connection \times Substrate	2,30	4.76	0.126

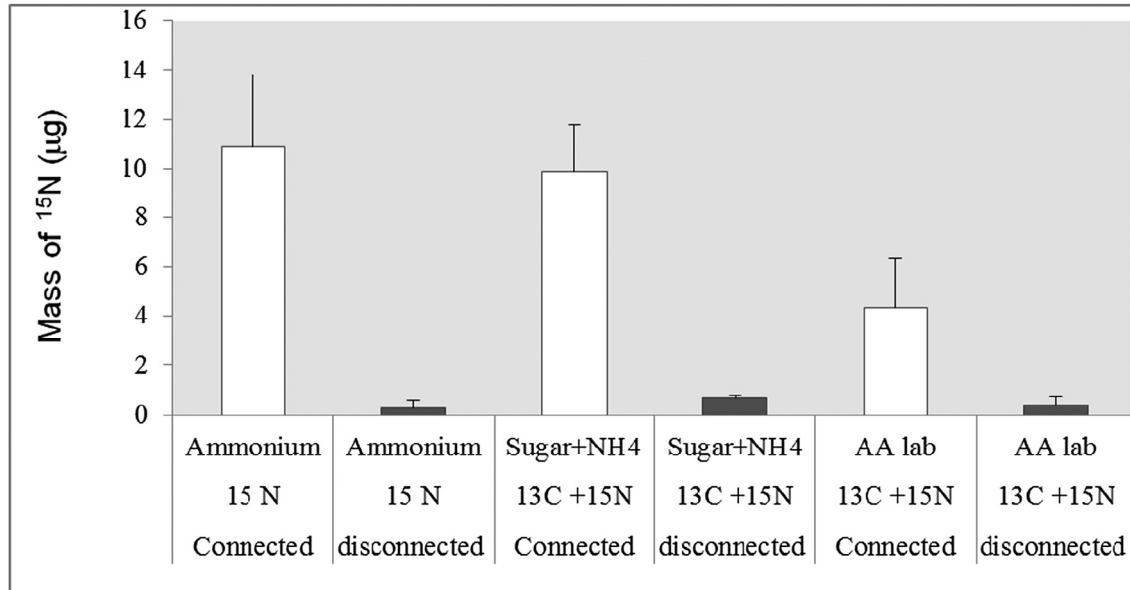


Fig. 9. Mass of ^{15}N connected with Carbon and Nitrogen sources associated with their fungal partners in the shoots of *A. laxiflora* plants. Routine ANOVA (TWO-WAY) and TMCT analysis were implemented for each-graph and difference between the mean mass (Table 5). $p < 0.05$.

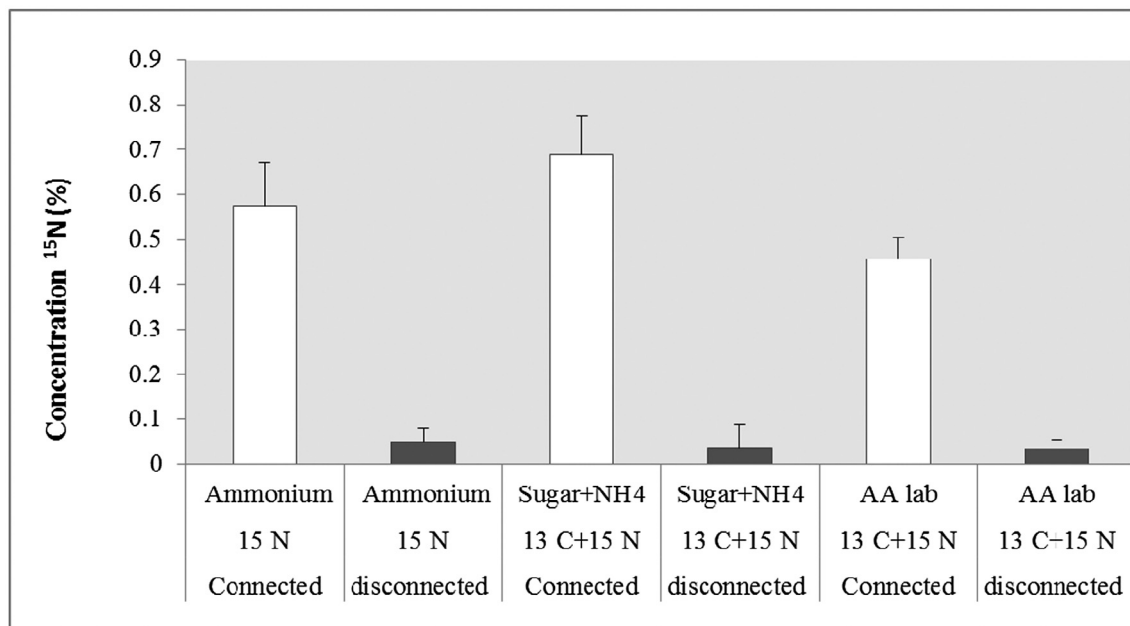


Fig. 10. *A. laxiflora* shoots of ^{15}N concentration of connected from the isotopically labelled Carbon-Nitrogen sources via using 2-way ANOVA analysis and TMCT in each graph, difference between mean of ^{15}N concentration (Table 6). $p < 0.05$.

et al. (2000) on *Catasetum fimbriatum* provides an organic and inorganic source for Nitrogen to orchid-plantlets referred as the largest growth rate for the non-mycorrhizal plants appeared with an amino-acids, showing these materials as essential basic sources of nitrogen for *C. fimbriatum*. This study is also referred as terrestrial protocorm is completely reliant on fungal partner for facilitation obtained of nitrogen. Previous studies were in the agreement with the current study results obtained through this study as large concentration of ^{15}N enrichment with NH_4CO_3 in addition of glucose in *A. laxiflora*, while, the major concentration of ^{15}N enrichment with an amino-acids in *A. palustris* shoots.

Both the carbon and nitrogen compounds are transferred through fungal partners have the positive impact on the establishment and growth of the orchid seedlings. Furthermore, displayed data above assists the concept of the further mycorrhizal fungi mechanisms in regulating through the germination of orchid-seeds. The carbon sources connected with nitrogen in an amino-acid formation to be considered. Presently, it has been established as amino-acids either in plant tissues or rhizosphere exudates, delivers the outstanding sources for carbon–nitrogen as the fungal partners. Fungal determination and the conversation of amino-acids into the sugars. Although, the small amount is moved into the plant. The transfer of ratio between the fungal to plant of carbon and nitrogen is more likely as solely depending on the nature of plant–fungal interfaces in the plant roots.

5. Conclusion

The current study concludes as the major interaction between carbon and nitrogen sources in regulating the growth and development tends to comprehend the dynamic relationship as an assimilation and transfer of carbon and nitrogen sources are influenced through the treatments. In an inquiry continues as the symbiosis status as a parasitic or mutualistic relationship even in the initial stages of autotrophy.

Declaration of Competing Interest

The author has declared that there are no conflicts of interest.

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