Studi Trent. Sci. Nat., 86 (2009): 67-70 ISSN 2035-7699

Atti XVIII Convegno Gadio 2008: *Un mondo che cambia: successioni ecologiche, invasioni biologiche ed alterazioni antropiche*
Sessione 4 - Poster

Molecular characterization of arbuscular mycorrhizal fungi in an agricultural soil and in potato roots

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SUMMARY - *Molecular characterization of arbuscular mycorrhizal fungi in an agricultural soil and in potato roots* - The symbiosis between plant roots and arbuscular mycorrhizal fungi (AMF) has been shown to affect both the diversity and productivity of agricultural communities. In this study, we characterized the AMF communities of *Solanum tuberosum* roots and of the bulk soil in two close areas of Italy, in order to verify if the land use practices selected any particular AMF with specificity to potato plants. The AMF large subunit rDNA genes (LSU) were subjected to nested PCR, cloning, sequencing and phylogenetic analyses. A hundred and eighty-three LSU rDNA sequences were analyzed and 8 monophyletic ribotypes, belonging to *Glomus* groups A and B, were identified. AMF communities differed between bulk soil and potato roots, as one AMF ribotype, corresponding to *Glomus intraradices*, was much more frequent in potato roots than in soils. Overall results, concerning the biodiversity of AM fungal communities in roots and in bulk soils from the two studied areas, suggested that potato were preferentially colonized by one AM fungal species, G. *intraradices*.

RIASSUNTO - *Caratterizzazione molecolare di funghi micorrizzici arbuscolari in un suolo agricolo e nelle radici di patata* - La simbiosi fra le piante ed i funghi micorrizici arbuscolari (AMF) può influenzare la biodiversità e la produttività delle comunità agricole. In questo studio abbiamo caratterizzato la comunità AMF in radici di *Solanum tuberosum* e nei suoli di due aree italiane allo scopo di verificare se le pratiche agricole abbiano contribuito a selezionare particolari ceppi di AMF con specificità verso le piante di patata. Il gene codificante la subunità maggiore dell'rDNA fungino (LSU) è stata utilizzato come stampo in una nested PCR, clonato, sequenziato ed sottoposto ad analisi filogenetica. Dall'analisi di 183 sequenze sono stati identificati 8 ribotipi monofiletici appartenenti al genere *Glomus* gruppo A e B. Differenze fra le comunità fungine dei suoli e delle radici di patata sono state evidenziate, ed un ribotipo, corrispondente a *G. intraradices*, era molto più frequente nelle radici di patata che nei suoli. I risultati ottenuti suggeriscono che le patate sono preferenzialmente colonizzate da una specie di AMF, *G. intraradices*.

Key words: arbuscular mycorrhizal (AM) fungi, genetic diversity, LSU rDNA, *Solanum tuberosum*, agricultural area
Parole chiave: funghi micorrizzici arbuscolari, diversità genetica, LSU rDNA, *Solanum tuberosum*, aree agricole

1. INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are obligate biotrophs, forming a mutualistic relationship with a broad range of host plant species, and among these crop plants (Preger *et al*. 2007). AM associations are generally considered to be non-specific, however, some studies show that preferential associations between plants and AM fungi might exist (Pivato *et al*. 2007). Agricultural management practices might affect the composition and diversity of AMF communities (Mathimaran *et al*. 2007) and in general, these have a negative impact on the AM association (Gosling *et al*. 2006). AMF abundance is reduced by P fertilization (Preger *et al*. 2007) and by cropping, mainly due to either mechanical disturbances by tillage or changes of host plants in crop rotation systems.

In our study we have analyzed an agricultural site renown for potato and onion productions, where potato has been grown for about 150 years and a 5-year crop rotation (wheat, potato, maize or onion, wheat and sugar beet) has been applied for at least 40 years. The indigenous AM fungal communities in the soil and in the roots of potatoes were detected by molecular methods and compared, in order to verify if the typical land use practices have selected particular AMF ecotypes with a host specificity to potato plants.

2. MATERIAL AND METHODS

2.1. Site of study, soil and potato sampling

The study was performed in an agricultural area

Tab. 1 - Mycorrhizal frequency (F%), mycorrhizal colonization (M%), arbuscule abundance (A%) and arbuscule abundance in the colonized area (a%) at the harvest. Different letters indicate statistically significant differences ($p < 0.05$) among treatments (along the columns of the table). From Cesaro *et al*. (2008).
Tab. 1 - Frequenza di micorrizazione (F%), colonizzazione micorrizica (M%), percentuale di arbuscoli (A%) e percentuale di arbuscoli nell'area colonizzata (a%) al momento della raccolta. Le differenti lettere indicano la significatività statistica ($p < 0,05$) relativa i trattamenti (lungo le colonne della tabella). Da Cesaro et al. *(2008).*

	F%	M%	A%	a%
CSN	30.7 ± 6.5 a	5.5 ± 2.1 a	4.9 ± 2.2 a	80.9 ± 9.2 a
CSS	16.7 ± 1.9 a	2.1 ± 0.7 a	1.8 ± 0.6 a	83.1 ± 5.5 a

around Castelnuovo Scrivia, Italy, (latitude 44°58'53" North, longitude 08°52'56" E, altitude 85 m). Considering the geographical (position relative to rivers and streams, orientation), chemical (pH, P content, organic matter content) and pedological (percentages of silt, clay, and sand) features of the territory, 2 homogenous areas of 40 km^2, called Castelnuovo Scrivia North (CSN; pH 7.98, P_i total 731 mg/kg, P_i available 18 mg/kg, organic matter 1.56%, silt 48.44%, clay 23.12%, sand 28.44%, USDA classification loam) and Castelnuovo Scrivia South (CSS; pH 8.00, P_i total 758 mg/kg, P_i available 17 mg/kg, organic matter 2.04%, silt 56.00%, clay 32.85%, sand 11.15%, USDA classification silty-clay-loam), were recognized, and in each of them four fields were selected. In each of these fields potatoes (*Solanum tuberosum L.*) were planted, in April 2005 and sampled in June 2005.

2.2. Assessment of root colonization

Mycorrhizal frequency (F%), mycorrhizal colonization (M%), arbuscule abundance (A%) and arbuscule abundance in the colonized area (a%) were evaluated microscopically according to Trouvelot *et al*. (1986). Results were statistically analysed by ANOVA followed by Fisher's PSLD test, with cut-off significance at $p < 0.05$.

2.3. DNA extraction, PCR amplification of a partial LSU rDNA region, cloning of PCR products and sequencing

For every area, the root samples were processed as described by Farmer *et al*. 2007 whereas for soil genomic DNA extraction were used the "Power soil DNA isolation kit" (MoBIO, USA) according to the manufacturer recommendations.

Genomic DNA was used for the amplification of a partial LSU rDNA region by a hemi-nested PCR, as described by Pivato *et al*. 2007, using the primer pair LR1 (van Tuinen *et al*. 1998) and FLR2 (Trouvelot *et al*. 1999) for the first amplification step, and LR1 and FLR4 (Gollotte *et al*. 2004) for the second one. PCR amplification products were cloned into the pCR4-TOPO vector (TOPO TA cloning kit for sequencing, Invitrogen, USA) and used to construct LSU rDNA soil and potato roots libraries (Pivato *et al*. 2007). Inserts were sequenced and the results were compared to known sequences using BLASTN (Altschul *et al*. 1997). Sequences have been deposited in the EMBL database, under the accession numbers AM947863 to AM947934.

2.4. Diversity analyses

Alignment of the sequences was performed using ClustalW 1.8.1 and optimised manually using the Se-Al v 2.0 software (University of Oxford). Phylogenetic analyses were performed using the neighbour joining (NJ) algorithm and using *Glomus versiforme* as outgroup.

To compare the richness and evenness of AM fungi in soils and in potato roots, the Shannon and Simpson diversity indices and the Shannon equitability index were applied.

3. RESULTS

The soil of both areas had similar pH and P_i, both total and available, values. They differed by the physical structure, soil from the CSN area being more sandy than soil of the CSS area. Also the chemical analyses of the soils of both areas revealed a high P_i concentration.

Colonization of the roots systems (%M) in areas CSN and CSS was low (Tab. 1), but the percentage of root fragments which were in contact with an AM fungus (F%) revealed the presence of active fungi in the soil. Although arbuscules were not abundant relatively to the whole root systems (A%), arbuscule frequency in the colonized portions of the roots was high (a%).

LR1-FLR4 PCR products obtained from potato roots or soil extract DNA were used to construct LSU rDNA libraries and from each of them 50 randomly selected clones were sequenced. No chimeric clones were detected by Blast analyses and the sequences obtained had a high similarity to LSU rDNA sequences from AM fungi. All sequences analyzed, corresponding to that expected for *Glomus* species. After phylogenetic analyses of the 183 sequences, 8 monophyletic ribotypes could be delineated on the basis of a bootstrap value of more then 980‰. Three groups clustered with already identified AM fungi, *G. mosseae* (group 1), *G. intraradices* (group 6) and *G. claroideum* (group 7). Five other groups did not cluster with any known Glomus sequence (Tab. 2). Most of the sequences obtained from the LSU rDNA originating from potato roots clustered with *G. intraradices* group (92% for CSN roots and 95% for CSS roots), whereas for the sequences obtained from the soils only 3 out of 48 (6.25%) and 2 out of 44 (4.5%) for soil CSN and CSS, respectively, clustered with *G. intraradices* group.

The Shannon and Simpson indices of diversity and evenness were applied in order to compare the richness and evenness of AM fungi in soils and in potato roots (Tab. 3). The number of expected ribotypes was higher in soils (5 ribotypes for soil CSN and 6 ribotypes for soil CSS) than in potato roots (2 ribotypes for potato roots CSN and CSS); D and H values in soils corresponded to higher sequence diversity (richness) than those observed in potato roots, whereas E_D referred to soils showed higher evenness than those of potato roots.

4. DISCUSSION AND CONCLUSIONS

In this study, the diversity of the AM fungal community was examined, by assessing the diversity of the LSU

Tab. 2 - Numbers of sequences showing the relative proportion of monophyletic groups originating from soils and from potato roots identified by the neighbour joining (NJ) algorithm and the bootstrap method with 1,000 replicates.
Tab. 2 - Numero di sequenze mostranti la proporzione relative dei gruppi monofiletici ottenuti dai suoli e dalle radici di patata identificate mediante l'algoritmo neighbour joining (NJ) ed il metodo bootstrap con 1.000 replicati.

	Group 1 *(Glomus mosseae)*	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6** *(Glomus intraradices)*	**Group 7** *Glomus (tunicatum/ claroideum)*	**Group 8**
Soil CSN	19	0	0	8	11	3	7	0
Soil CSS	5	2	2	21	12	2	0	0
Potato CSN	0	0	0	0	4	44	0	0
Potato CSS	0	0	0	0	0	41	0	2

rDNA (van Tuinen *et al*. 1998), in two soils with different features and where a crop rotation was applied.

Eight monophyletic ribotypes defined on the basis of the bootstrap level (980 ‰), were identified. All sequences belonged to the genus *Glomus* (group A and B) (Schwarzott *et al*. 2001) usually dominating the AMF communities in European agricultural soils (Mathimaran *et al*. 2005). This number was low however the number of sequences analysed appeared to be sufficient to describe the AM fungal diversity.

The genetic diversity of AM fungi associated with CSN and CSS soils was characterized and five monophyletic ribotypes were identified in soil CSN, three of them belonging to already known AM fungi. Similarly, three out of six of monophyletic ribotypes detected in soil CSS were already known. The low AMF soil biodiversity was likely due to the typical agricultural management practices of the Castelnuovo Scrivia area, as an inverse relationship between management intensity and AMF diversity has been observed several times (Preger *et al*. 2007).

The overall levels of root colonization in plants from CSN and CSS soils were rather low. In contrast, the number of root fragments in contact with an AM fungus (F%) revealed the presence of active fungi in the soils. However, the high a% value suggested an active AM symbiosis in the mycorrhizal fraction of the root system. Agricultural management practices and the high P_i concentration of both areas could explain the relative low level of mycorrhization of the potato roots (McArthur & Knowles 1992), although the growth time was sufficient for the establishment of extensive colonization.

Tab. 3 - Shannon and Simpson diversity indices and Shannon equitability index to compare soil and root richness and evenness. H= Shannon diversity index, EH= Shannon equitability and D= Simpson diversity index. From Cesaro *et al*. (2008).
Tab. 3 - Indici di diversità di Shannon e di Simpson ed indice di equitability di Shannon per confrontare la richness e la evenness dei suoli e delle radici. H= Shannon diversity index, EH= Shannon equitability and D= Simpson diversity index. Da Cesaro et al. *(2008).*

	Shannon's indices		Simpson's index
	H	E_H	D
CSN soil	1.176	0.731	0.308
CSS soil	1.376	0.768	0.227
CSN potato	0.287	0.414	0.777
CSS potato	0.188	0.271	0.728

The genetic diversity of AM fungi associated with CSN and CSS potato roots was characterized and in both root samples two monophyletic ribotypes were identified, one of which belonging to an already known AM fungus, *G. intraradices*. In particular, 92% and 95% of the sequences identified from CSN and CSS potato roots, respectively, corresponded to *G. intraradices*. Even if this fungus was not the most abundant in the soils, it was largely dominating in potato roots. Similar data, but not as evident as in our results, were obtained in root nodules (Scheublin *et al*. 2004), but in this case only 63% of the obtained sequences corresponded to *G. intraradices*. The reasons for this association are not known, but previous observations showed that changes in AMF community composition occurred after N fertilization (Eom *et al*. 1999) and that *G. intraradices* was related to nitrogen-enriched soils (Jumpponen *et al*. 2005). The same results were obtained for potato roots planted in both soils despite their different pedological features.

Also diversity, estimated by Shannon and Simpson indices in potato roots and in the soils, presented some variations; in particular, the AM fungal sequences were more evenly distributed in soils in comparison to potato roots, and Shannon equitability indices for the two soils showed a higher evenness than those for roots. The different biodiversity of the AM fungal communities associated with two soils and with potato plants detected in our study further suggest that the tested plants selected a specific AM fungal genotype. In addition, also a more sensitive method confirmed our cloning results (Cesaro *et al*. 2008) suggesting a specific interaction plant-fungus.

Several factors could affect the development of AMF communities in roots and soil, for example seasonal changes (Sýkorová *et al*. 2007) as previously shown for *G. intraradices*, possibly in relation to fluctuations in N mineralization (Santos-Gonzáles *et al*. 2007). Photosynthate allocation, which in potato is affected by temperature, light intensity, N availability and plant developmental stage (Sweetlove & Hill 2000), might also play a role by affecting C availability for the mycobionts. Finally, early colonization by one AM isolate might prevent further colonization by other fungi, a

phenomenon known as autoregulation (Vierheilig 2004).

In conclusion, we propose that the typical land use practices used in Castelnuovo Scrivia have led to a very specific interaction between *G. intraradices* and potato plants. This suggests that the plant species and the agricultural practices used in this area have exerted a selective pressure, and that some form of specificity between AM fungal and plant genotypes exists. The reasons of this specific association between *G. intraradices* and *S. tuberosum* require further investigation.

ACKNOWLEDGEMENTS

This work was partly funded by Progetto Alfieri "I prodotti tipici agroalimentari dell'Alessandrino e la loro comunicazione a livello nazionale e internazionale" (Fondazione Cassa di Risparmio di Torino). The authors wish to thank Dr. Elisa Gamalero and Prof. Stefania Biondi for critical reading of the manuscript.

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