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Fungal colonization improved growth and modulated the expression of myrosinases in black cabbage

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Abstract: The role of beneficial microorganisms, such as mycorrhizas, in improving the resistance to environmental stress of colonized plants is well-known. Plants of Brassicaceae family are of large economic importance, especially for the synthesis of anticarcinogenic compounds such as glucosinolates and their derivatives isothiocyanates. The endophyte fungus *Piriformospora indica* is able to colonize them and improves their growth and response to environmental stress. However, no information are available on the impact of colonization on glucosinolate metabolism. In this work, colonization of black cabbage (*Brassica oleracea* cv. Acephala sabellica) is reported as well as the effects on plant growth and on the expression of myrosinase encoding genes, the isothyocianate producing enzymes. Results indicate that *P. indica* successfully colonized black cabbage as validated by the expression of the marker gene Ptef1. Colonized plants showed increase of biomass weights and shoot length respect to the uncolonized plants and a decrease of myrosinase gene expression. This last finding indicates that *P. indica* might affect the resistance against biotic stress of black cabbage.

1. Introduction

Over the last 20 years, low-input and organic agriculture has increased worldwide to preserve agroecosystem functionality (Postma-Blaauw *et al.*, 2010). The main point of such an agriculture is a systemic approach to integrate sustainable yield and crop quality together with high-energy efficiency and low environmental impact (Pimentel *et al*., 2005; Moonen and Bàrberi, 2008). In the framework of this view, the natural roles of microorganisms, such as arbuscular mycorrhizas in improving soil fertility have gained a growing interest for the use of such microrganisms as ecosystem engineers and biofertilizers (Fitter *et al*., 2011). Although arbuscolar mycorrhizal fungi normally infect most species of plants, some plants taxa do not usually form generally recognisable mycorrhizas. Among them, the family of Brassicaceae have been considered to be nonmicorrhyzal plants (Lambers and Teste, 2013), probably because their roots released anti-fungal metabolites such as isothiocyanates in the surrounding environment (Tester *et al*., 1987). Isothiocyanates are produced by hydrolization of glucosinolates that are a group of secondary metabolites present in Brassicaceae (Halkier and Gershenzon, 2006).

The endophyte fungus *Piriformospora indica* (*P. indica*), a basidiomycete of the order Sebacinales, was isolated from the Indian Thar desert in 1997 (Varma *et al*., 1999). *P. indica* has received a great attention over the last few decades due to its ability to promote plant growth, protection and stress tolerance in colonized plants (Verma *et al.*, 1998; Banhara *et al*., 2015). *P. indica* is similar to arbuscular mycorrhizal fungi, but it is a facultative symbiont and can be easily grown on various synthetic media. Likewise, *P. indica* has a wide host range, colonizes the host roots, grows inter and intracellularly, and forms pear-shaped chlamydospores within the cortex, improving the growth of many plant species, enhancing nutrient uptake, enabling plants to cope with environmental conditions, and to survive under abiotic stresses. It also confers resistance to toxins, pathogenic microorganisms, and increases seed biomass yield (Oelmuller *et al.*, 2009). Among others, *P. indica* is able to colo-

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nize plants of Brassicaceae family (Sherameti *et al.*, 2005) and improves their growth and response to environmental stimuli. *P. indica* triggered local and systemic root responses in *Arabidopsis thaliana* (Pedrotti *et al*., 2013). In Chinese cabbage (*Brassica rapa*), it has been reported that *P. indica* colonization confers drought tolerance stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized Ca(2+)-sensing receptor (CAS) protein in the leaves (Sun *et al*., 2010). Black cabbage, (*Brassica oleracea* cv. acephala sabellia) a variety of kale largely used in Italian cuisine, especially in Tuscany, where has been grown for centuries (Appleman *et al*., 2008), is generally considered a nonmicorrhyzal plants (Lambers and Teste, 2013). In this work, with purpose to assess whether *P. indica* colonizes black cabbage and to study the colonization effects on this cultivar, seedlings were inoculated with *P. indica*; morphological parameters and the expression of myrosinase encoding genes were studied.

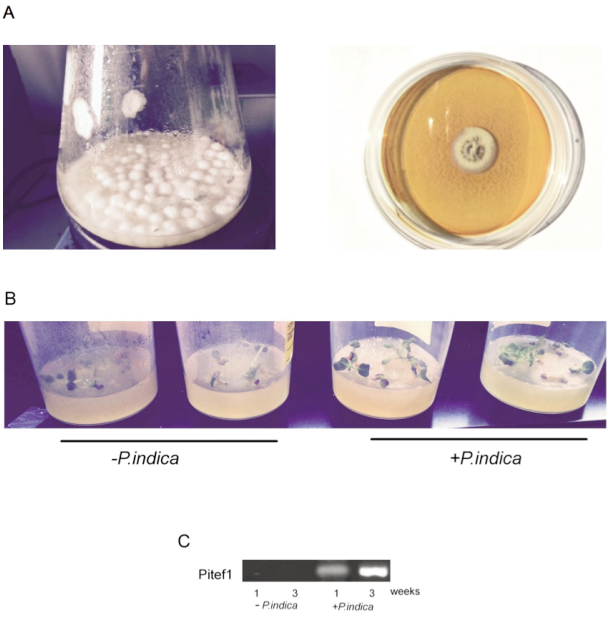

Fig. 1 - *P. indica* grown in liquid (left panel) or agar medium (right panel) (A); control (- *P. indica*) or colonized (+ P. indica) black cabbage seedlings grown on 1.5% agar (B); Pitef1 expression in *-P.indica* (left panel) or + *P.indica* plants (right panel) (C).

2. Materials and Methods

Growth conditions of plants and fungus, and estimation of plant growth

Seeds of *Brassica oleracea* L. ssp. *oleracea* convar *acephala* (DC.) Alef. var. *sabellica* L. were surface-sterilized with 75% alcohol three times for 10 min, and then placed on a Petri dish containing sterilized water. Plates were incubated at 22°C under continuous illumination (for seed germination). After 7 days, seedlings were transferred in Petri dish plates with solid (1.5% agar) complete medium (CM) (Pham *et al*., 2004). Six seedlings were used per plate.

Piriformospora indica growth conditions

P. indica cultures, DSM11827, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany (Lahrmanna *et al*., 2013) were propagated at 28°C in liquid CM for two days than plated in agar CM (Fig. 1A). The amount of 200 mg of fungal mycelium were used to colonize the seeds. 0.1 ml of CM medium containing fungal mycelium were positioned 1 cm away from each seedling. The same amount of autoclaved mycelium was used as control. Plant growth was monitored day by day. Histograms report biomass weight and shoot length as mean±SD. The statistical significance of differential findings between samples was determined by ANOVA using NIA software; $p<0.05$ was considered statistically significant.

RNA extraction and genes expression Pitef1 expression

Brassica leaves were disrupted by liquid nitrogen and then suspended in the double volume of PBS. Total RNA extraction and cDNA synthesis were performed from 50 mg of lised leaves samples, modifying the protocol of the Taqman Gene Expression Cells-to-CT TM Kit (Applied Biosystems) as reported in Podda *et al.* (2014). Two µL of the cDNA were used for sqRT-PCR amplification performed with GoTaq Green Mastermix (Promega, USA). The following standard thermal profile was used for all PCRs: 94°C for 3 min; 35 cycles of 90°C for 30 s, 59°C for 40 s, and 72°C for 40 s; 72°C for 7 min as final extension. PCR products were separated by 1% agarose gel electrophoresis and stained with GelRed (Biotium). cDNA fragments were purified from gels and sequenced by BMR-Genomics (Italy). Transcript levels were measured by Scion Image program and normalized with the constitutive reference actin gene (Wang *et al*., 2016). Three independent biological replicates were used. In order to verify the colonization level, the presence of *P. indica Transcription Elongation Factor* Pitef (Butehorn *et al.*, 2000) was tested in the *P. indica* leaves before or after fungal colonization.

The following primers have been used:

	F 5'	Rew 5'
Pitef1	ATTGCCTGCAAGTTCTCCGA	CTTCGTAACCTTGCCACCCT
TGG1	TCTTAACGTGTGGGATGGCT	CCTCCTTTGTTCACTCCCCT
TGG2	AGATGTGCTGGACGAACTCA	CGGCGTAACAGGTAGGATCA
PEN2	GCATCATCATCCAACAGCGT	ACGCCTTGATCAGTTCTCCA
Actin	AATGGTACCGGAATGGTCAA	AGTTGCTCACAACACCATGC

3. Results and Discussion

P. indica growth and black cabbage colonization

In order to evaluate the effects of *P. indica* colonization in black cabbage, the protocol used by Dolatabadi and Goltapeh, (2013) has been optimized for this kale variety. The fungus *P. indica* was grown in liquid medium (Fig 1 A, left panel) and then transferred on agar complete medium (Fig 1 A, right panel). Then 7 week-old black cabbage seedlings were inoculated with *P. indica* mycelium in sterilized conditions in tubes. To validate the successfully colonization, the expression of *Pitef1* was assessed as the gene has been demonstrated to be useful for estimating the amount of active mycelium introduced in seedlings (Butehorn *et al*., 2000). A strong expression of *Pitef1* was observed in leaves of colonized seedlings of black cabbage one and three weeks after fungal inoculation whereas no transcript was observed in not-colonized seedlings (Fig. 1C).

Evaluation of black cabbage growth parameters

The effects of colonization on growth parameters, biomass weights and shoot lengths, were measured in the inoculated plants in the first three weeks of growth. Colonization by P*. indica* resulted in a rapid enhancement of about 30% of root and shoot biomass respect to the not colonized plants, just after one week from the inoculation (Fig. 2 A, B). Results are in agreement with those reported by Dolatabadi and Goltapeh, (2013) who found that *P. indica* and *Sebacina vermifera* improved the growth of *B. oleracea* and other brassicaceae plants. Satheesan et al., (2012) reported improved growth of *Centella asiatica* after inoculation by *P. indica.*

Expression of TGG1, TGG2, PEN2 in the leaves

An increase of glucosinolates was found within ten days from germination in black cabbage. Glucosinolates are secondary metabolites present in Brassicaceae (Yi *et al.*, 2015). When plants are damaged due to insect herbivore attack, glucosinolates are hydrolyzed quickly with myrosinase (β-thioglucoside glucohydrolase or thioglucosidase) resulting in production of isothiocyanates, thiocyanates, nitriles and others compounds (Bones and Rossiter, 2006; Hopkins *et al.*, 2009). No information are available in the literature on the modulation of glucosinolate by products in the leaves during fungal colonization.

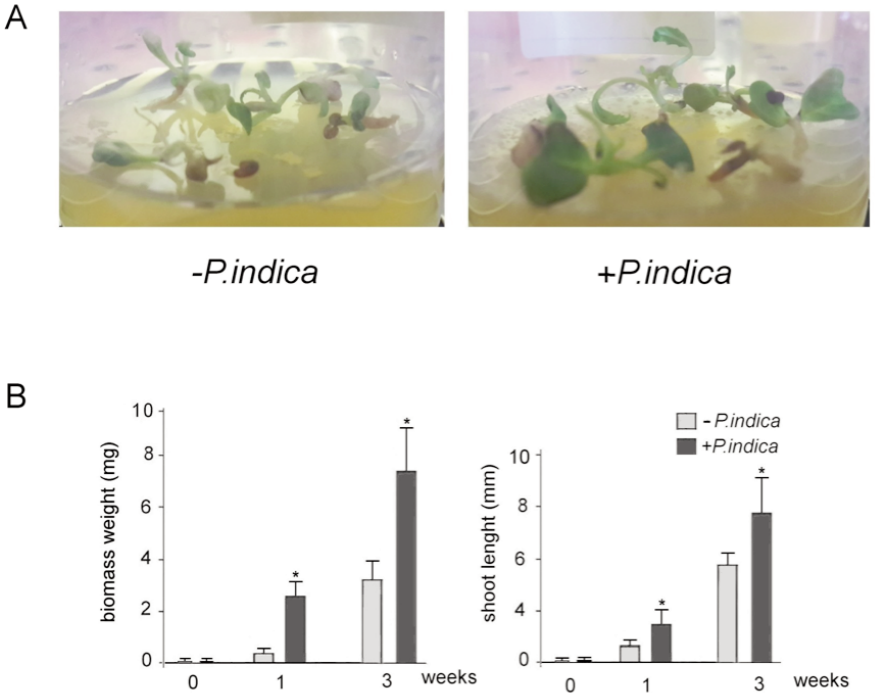

Fig. 2 - Influence of *P. indica* on black cabbage growth parameters during three weeks from the inoculation. Picture of the plants after one week from the inoculation (A); Biomass weight (mg) or shoot length (mm), at 0, 1 and 3 weeks after *P. indica* colonization (B).Values are the mean of ten independent experiments for each condition (control or inoculated) ±SD. Asterisk means significant difference at $p \leq 0.05$.

Thus, in this work the expression of TGG1 and TGG2, which encode myrosinases hydrolysing aliphatic glucosinolates or PEN2, encoding the enzymes hydrolysing indole glucosinolates was evaluated. Intriguingly, a decrease of TGG1, TGG2 and PEN2 expression was observed at three weeks of colonization (Fig. 3). Similar results have been reported by Witzel *et al*. (2015) in *Arabidopsis thaliana* infected by *Verticillium longisporum*. As the glucosinolate-myrosinase system is relevant for defence against insect-herbivore (Winde and Wittstock, 2011), the decrease of the expression of the genes relative to this pathway, is of particular importance and should be further investigated for extensive periods. Although *P. indica* colonization improves plant growth and resistance to abiotic stress (Sun *et al*., 2010), a negative impact on defence response pathway might increase the susceptibility of colonized black cabbage against biotic stress.

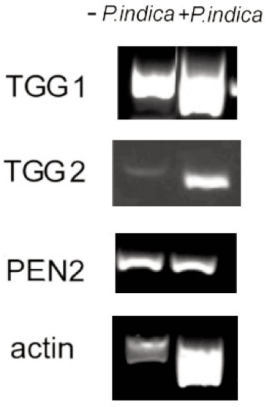

Fig. 3 - Influence of *P. indica* on black cabbage myrosinases. Analysis of TGG1, TGG2 and PEN2 expression in leaves three weeks after colonization by sqRT-PCR. Actin was used as reference gene.

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