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Biocontrol of *Rhizoctonia solani* disease and biostimulant effect by microbial products on bean plants

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Abstract

Microbial products containing a mixture of fungi and bacteria (EM Bokashi® 2-fi and EM-5 Sutociu® characterised by plant biostimulant activity), *Trichoderma harzianum* T22 (biofungicide) and the antagonist fungus *Trichoderma* sp. TJ40 were tested for efficacy against *R. solani* disease and for their biostimulant effects on bean plants, in growth chamber experiments, and for their direct effect on the pathogen growth, through *in vitro* experiments. In growth chamber experiments, EM-5 Sutociu was applied to seed (Sut/Se), substrate (Sut/S) and leaf (Sut/L) many times, EM Bokashi 2-fi to substrare (Bok/S) once and combined with Sut, T22 and TJ40 were applied once to substrate. The pathogen was inoculated to substrate at seeding time (first experiment) or at seedling phase (second experiment). Under our experimental conditions, Bok/S+Sut/S+Sut/L, Sut/S+Sut/L, Sut/Se+Sut/S+Sut/L and T22, in the first experiment, and all treatments, with the exception of Bok/S applied alone in the second experiment, gave significantly disease severity reduction and increase of dry weight and leaf area with respect to the infected control. The TJ40 treatment reduced both disease incidence and disease severity only in the second experiment. In the experiment on the biostimulant effect, T22, Bok/S+Sut/S+Sut/L, Sut/S+Sut/L and Sut/Se+Sut/S+Sut/L showed significantly increases of both dry weight and leaf area. The direct effect of the treatment with T22, TJ40, Bok and Sut on *R. solani* growth *in vitro* was studied with two methods, submerged colony (SC) and well diffusion (WD) assays. The pathogen growth was completely inhibited by *Trichoderma* T22 in both assays, by *Trichoderma* TJ40 in a range of 80-50 % in SD assay, and 50-30 % in WD assay and slightly inhibited or not inhibited by Bok and Sut.

Keywords: *Rhizoctonia solani;* antagonistic microorganisms; plant biostimulants; biocontrol

Riassunto

Rhizoctonia solani Kühn, è un fungo basidiomicete ad *habitat* terricolo, diffuso in tutto il mondo su numerose piante coltivate e causa di significative perdite di produzione. Tra le varie colture, il fagiolo è altamente suscettibile alla malattia causata da *R. solani.* Il controllo del patogeno, così come di altri patogeni terricoli, può essere ottenuto con la disinfezione del suolo. Tuttavia, in Europa, i prodotti chimici per la difesa delle piante dalle malattie sono stati oggetto di restrizioni d'utilizzo che hanno comportato l'esclusione di molti principi attivi contro i patogeni compresi quelli terricoli. Di conseguenza, molti studi sono stati

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finalizzati alla ricerca di mezzi alternativi a quelli chimici, come ad esempio i microrganismi. Alcuni di questi, definiti "antagonisti", sono componenti di bioagrofarmaci autorizzati per la difesa delle piante, ma possono anche essere contenuti in prodotti dotati di azione biostimolante per le piante. In questa ricerca, è stata studiata, in esperimenti in cella climatica, l'efficacia contro *R. solani* e l'azione biostimolante su piante di fagiolo di prodotti microbici contenenti funghi e batteri (EM Bokashi® 2-fi and EM-5 Sutociu® caratterizzati da un'azione biostimolante delle piante), *Trichoderma harzianum* T22 (un noto biofungicida) e l'antagonista fungino *Trichoderma* TJ40. EM-5 Sutociu è stato somministrato ai semi (Sut/Se), al substrato (Sut/S) e alle foglie (Sut/L) più volte, EM Bokashi 2-fi al substrato (Bok/S) singolarmente e in combinazione con Sut; T22 and TJ40 sono stati somministrati una volta ciascuno al substrato. Il patogeno è stato inoculato nel substrato al momento della semina (primo esperimento) oppure all'emergenza nella fase di piantina (secondo esperimento). Nelle nostre condizioni sperimentali, Bok/S+Sut/S+Sut/L, Sut/S+Sut/L, Sut/Se+Sut/S+Sut/L e T22, nel primo esperimento, e tutti i trattamenti ad eccezione di Bok/S somministrato da solo, nel secondo esperimento, hanno ridotto significativamente la gravità della malattia, incrementato il peso secco delle piante e l'area fogliare rispetto il controllo infetto. TJ40 ha ridotto sia l'incidenza di malattia sia la gravità solo nel secondo esperimento. Nell'esperimento sull'azione biostimolante, T22, Bok/S+Sut/S+Sut/L, Sut/S+Sut/L e Sut/Se+Sut/S+Sut/L hanno mostrato di aumentare significativamente sia il peso secco che l'area fogliare. E' stato inoltre studiato *in vitro* l'effetto diretto di T22, TJ40, Bok e Sut sulla crescita di *R. solani* con due metodi: "submerged colony" (SC) e "well diffusion" (WD). La crescita della colonia del patogeno è stata completamente inibita da T22 in entrambi i metodi, da TJ40 in un range di 80-50 % nel saggio con il metodo SD, e in un range di 50-30 % nel saggio WD e leggermente inibita o non inibita da Bok and Sut.

Parole chiave: *Rhizoctonia solani*; microorganismi antagonisti; biostimolanti delle piante; lotta biologica

Introduction

Rhizoctonia solani Kühn, teleomorph *Thanatephorus cucumeris* (Frank) Donk, is a soil-borne basidiomycete fungus globally-distributed. This fungus survives and overwinters in soil as mycelium or as sclerotia in plant debris and can infect plants during the growing season. This pathogen employs a necrotrophic lifestyle on numerous cultivated plant species causing significant crop losses (Parmeter, 1970; Sneh et al., 1991). Among the various crops, bean is highly susceptible to stem, hypocotyl and root rot disease by *R. solani.*

As for almost all soil-borne pathogens, the control of *R. solani* could be obtained by soil disinfestation, an important practice in horticultural intensive systems. However, nowadays, effective chemical fumigants for soil disinfestation, such as methyl bromide, chloropicrin, dazomet and 1,3-dicloropropene, are no longer included in the European list of approved substances. In Italy, very few chemicals are allowed for use on bean crops; among them are fumigants with some fungicide effect, such as methyl isothiocyanate generators, metham sodium and metham potassium, and non-fumigant fungicides for seed disinfestation, such as thiram alone or in combination with carboxin. Recently, some microbial products are commercialized as "plant protection products" or "pesticides" for soil application. All plant protection products in the European Union, and therefore in Italy, are regulated by the Regulation (EC) No 1107/2009. They are contained in a list of approval under the cited EC Regulation (EU Pesticide database, 2015). Among these products, those sold in Italy for soil application against plant diseases are based on fungi (*Trichoderma harzianum*, *T. asperellum* and *T. gamsii* and *Coniothyrium minitans*), or bacteria (*Streptomyces* sp.), and can be used both in integrated pest management (IPM), which is mandatory in Italy since 1 January 2014, both in organic agriculture. Among fungi, *Trichoderma* spp. are the most

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widely species studied for a long time (Tronsmo & Hjeljord, 1998) and, colonizing roots, can enhances root growth and development, crop productivity, resistance to biotic and abiotic stresses and the uptake and use of nutrients (Harman et al., 2004; Matsouri et al., 2010), acting as a plant biostimulant.
Plant biostimulants are defined by the EBIC (European Biostimulants Industry) as follows: "Plant biostimulants contain substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality. Biostimulants have no direct action against pests, and therefore do not fall within the regulatory framework of pesticides" (European Biostimulants Industry, 2012a). Biostimulants also include products with some nutrients, but their effect on plant growth promotion is not through direct fertilization. In fact, the EBIC (2012b) cites "Biostimulants operate through different mechanisms than fertilisers, regardless of the presence of nutrients in the products". Brown & Saa (2015) hypothesized that biostimulants are beneficial for plant productivity by interacting with plant signalling processes thereby reducing negative plant response to stress, and Veronesi et al. (2009) demonstrated that some biostimulants are able to enhance the plant defence mechanisms against biotic stresses. Moreover, biostimulants can potentially act against plant pathogens since they also contain microorganisms such as fungi (e.g. *Trichoderma* sp.) and bacteria (e.g. *Bacillus subtilis*). Therefore, based on their characteristics, biostimulants may have an important role in the integrated production.
Based on the above considerations, and also on the absence of commercial cultivars of bean resistant to the disease caused by *R. solani,* the objectives of this study were to investigate i) the role of microbial products on the disease control, and their biostimulant effect on bean in growth chamber experiments, and ii) their direct effect on the pathogen growth through *in vitro* experiments.

Materials and Methods

Plant material, pathogen and natural compounds

Seeds of bean (*Phaseolus vulgaris* L.) cv. Borlotto (Semencoop a.r.l, Martorano di Cesena, FC, Italy) were employed. Before seeding, 200 seeds randomly selected were analysed to verify the absence of *R. solani* natural infection. Seeds were firstly observed visually, to exclude the presence of brown lesions, then superficially disinfected with sodium hypochlorite solution (NaOCl, 2% available chlorine), and plated on potato dextrose agar (PDA, 3.9 %, Difco, Detroit, MI, USA) added with streptomycin sulphate (100 mg/L). Plates were incubated at 25 °C in the dark for 10 days.
The pathogen *R. solani* (Rs) (Fig. 1) was obtained from bean seedlings showing symptoms of post-emergence damping-off and brown lesions on the hypocotyl. *Rhizoctonia solani* was identified based on morphological features by examining hyphal branching septal, pore type and cellular nuclear number close to the tips of young hyphae, after 2 days of growth on alkaline water agar plus 100 µg/ml streptomycin sulphate (Gutierrez et al., 1997, modified), followed by staining with aniline blue. Colonies of the fungus also showed sclerotia of irregular shape and monilioid cells after 21 days growth on PDA. This fungus was pathogenic on bean cv. Borlotto in a previous experiment and caused characteristic crown and root rot symptoms. The pathogen was kept on PDA at 4°C until use.
The tested compounds were all based on microorganisms and are listed in Table 1. EM Bokashi® 2-fi and EM-5 Sutociu® are commercialized as biostimulants, Trianum-P is a biofungicide registered by Italian Health Ministry for the application against soil-borne pathogens on various crops. Moreover, the *Trichoderma* sp. J40 strain (Fig. 2), kindly supplied by CRA - Centro di Ricerca per le Colture Industriali di Bologna (Italy), was tested.

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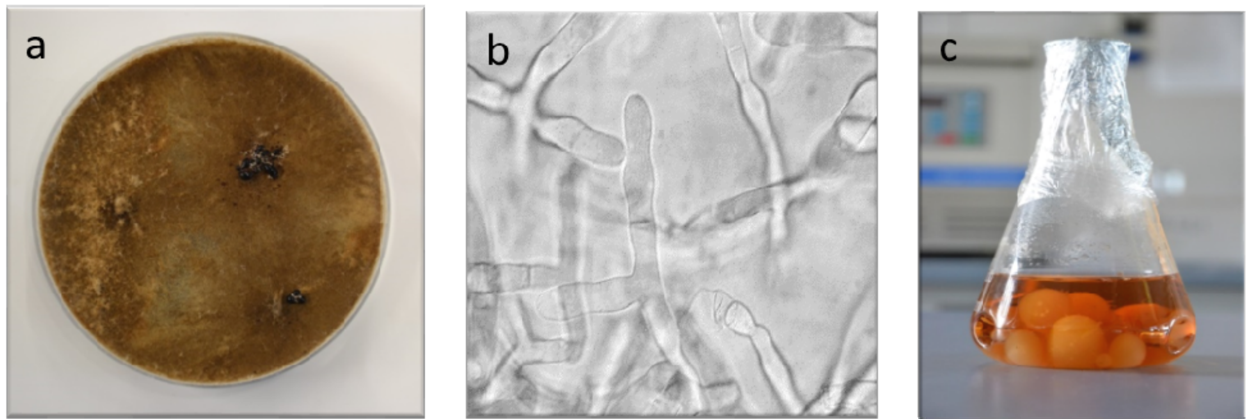

Fig. 1 - *Rhizoctonia solani*: colonies grown on PDA for 10 days (a), hyphae with typical ramifications and monilioide cells at light microscope (b) and mycelium grown in PDB for 10 days in an orbital shaker (c).
Rhizoctonia solani: colonie cresciute su PDA per 10 giorni (a), ife con tipiche ramificazioni e cellule monilioidi al microscopio ottico (b) e micelio cresciuto in PDB per 10 giorni in un agitatore orbitale (c).

Fig. 2 - Colony of *Trichoderma* sp. J40 grown on PDA for 7 days.
Colonia di *Trichoderma* sp. J40 cresciuta su PDA per 7 giorni.

Growth chamber experiments

All growth chamber experiments were carried out at 25 ± 1°C, 84 88 % relative humidity, 12-h photoperiod (5000 lux) using a plant growing substrate constituted by loamy soil, peat moss and sand (2:1:1 v:v:v). The substrate was heat-treated at 120°C for 30 min on two consecutive days, and then cooled before the use. Before seeding, the bean seeds were first surface-disinfected with NaOCl (2% available chlorine) for 5 min, rinsed twice in sterile distilled water and dried on sterile paper in a laminar flow cabinet. Seeds were sown in 12 × 10 × 6 cm pot at the rate of 20 seeds per pot. Each pot represented a replicate. The experiments were laid out in completely randomized design with three replicates of each treatment. Each experiment was repeated twice.

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Tab. 1 - Microbial products, active ingredients, dosage, site and symbol, and time of application in greenhouse experiments.
Prodotti a base di microrganismi, principi attivi, dosi, sito d'applicazione e relativo simbolo e momento di trattamento.

Microbial products (Company)	Active ingredients	Dosage	Site	Symbol	Time/application
EM Bokashi® 2-fi (Punto EM S.r.l., Sanremo, IM, Italia)	Fungi and bacteria[a]	20g/L	substrate	Bok/S	at seeding by irrigation
EM-5 Sutociu® (Punto EM S.r.l., Sanremo, IM, Italia)	Fungi and bacteria[b]	1 ml/L	seedc	Sut/Se	
		5 ml/L[d]	substrate	Sut/S	at seeding by irrigation
		5 ml/L[e]	leaves	Sut/L	twice a week from emergence by spraying[f]
		20 ml/L[g]	leaves	Sut/L	every day by irrigation[h]
Trianum-P (Koppert Italia S.r.l, Bussolengo, VR)	*Trichoderma harzianum* T22	1.5 g/3L water/m^2	substrate	T22	at seeding by irrigation
-	*Trichoderma* sp. J40	5×10^6 cfu	substrate	TJ40	at seeding by irrigation

[a]*Lactobacillus plantarum* (1×10^6 cfu/ml), *L. casei* (1×10^6 cfu/ml) and *Streptococcus lactis* (1×10^6 cfu/ml), *Rhodospeudomonas palustris* (1×10^3 cfu/ml) and *Rhodobacter spaeroides* (1×10^3 cfu/ml), *Saccharomyces cerevisiae* (1×10^5 cfu/ml) and *Candida utilis* (1×10^5 cfu/ml), *Streptomyces albus* (1×10^4 cfu/ml) and *S. griseus* (1×10^4 cfu/ml), *Aspergillus oryzae* (1×10^3 cfu/ml), *Penicillum* sp. (1×10^3 cfu/ml), *Mucor hiemalis* (1×10^4 cfu/ml), *Trichoderma harzianum* (1×10^4 cfu/ml), *T. viride* (1×10^3 cfu/ml) added to bran (D. Prisa, personal communication); [b]same microorganisms of [a] suspended in liquid substances such as grappa and allium extract; [c]treated for 12 hrs in liquid suspension; [d]150 ml per pot; [e]150 ml per pot, until the suspension has dripped over the soil; [f]until plants were symptomless; [g]50 ml per pot, until the suspension has dripped over the soil; [h]from disease symptom appearance.

Biocontrol activity

The substrate was inoculated with Rs at the rate of 3 g mycelium/L of substrate at sowing time and at seedling phase time (6 days after seeding), in order to simulate an early or a late pathogen attack. For the inoculum preparation, ten 1-cm2 discs from the actively growing colonies of Rs on PDA were inoculated in each 300 ml Potato Dextrose Broth (PDB, 2.4%, Difco, Detroit, MI, USA) conical flask. The flasks were gentle shaken at 100 rpm for 7 days at 23- 25 °C in the dark. The mycelial mass (Fig. 1) was collected by filtration through Whatman n. 1 filter paper, rinsed with distilled water, weighted and finely homogenized in a sterile Ultra-TURRAX® (IKA, Germany) blender for 30 sec.
Microbial compounds were applied following instruction of manufacturer (Table 1), except for *Trichoderma* sp. J40 that was distributed into substrate as conidial suspension in Ringer's solution (Sigma-

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Aldrich Co, St. Louis, MO, USA)) (5 × 106 cfu/ml). For each substrate and leaf treatment, a volume of 150 ml of each EM product (20 ml of Sut/L only in presence of disease symptoms) was distributed in each pot at the dose and times indicated in Table 1. For seed treatment, seeds were immersed in an aqueous suspension of Sut/Se for 12 h. Infected control received the same volume of sterile distilled water.
Three weeks after pathogen inoculation, the plants were carefully collected and washed under running water. The disease incidence (DI) was determined by counting plants with disease symptoms on the total of plants. For disease severity (DS), plants were counted and divided into four classes of severity (Fig. 3) as follows: 0 = no lesions, 1 = lesions 2.5 mm, 2 = lesions 2.5–5 mm; 3 = lesions > 5 mm and girdling plant (modified from Cardoso and Echandi, 1987). Disease severity index was calculated for each pot by the following formula: [((plant number in class 1) + 2 (plant number in class 2) + 3 (plant number in class 3))/total plant number] × 100/3.
Samples of root and foot 1 2 cm long from plants with Rs disease symptoms were surface disinfected with 5% NaOCl, rinsed in sterile distilled water and transferred to Petri dishes containing PDA supplemented with 100 mg/L of streptomycin sulphate (Sigma - Aldrich Co.). After incubation at 25°C in the dark for 10 days, the presence of Rs was confirmed using a light microscope (Carl Zeiss mod. ZM, Germany) at × 300 magnification.
For each pot, plant leaf area was measured with the LI-3100 Area Meter (LI-COR Bioscience, USA) and total plant dry weight was determined after oven drying at 80 °C for 24 h.

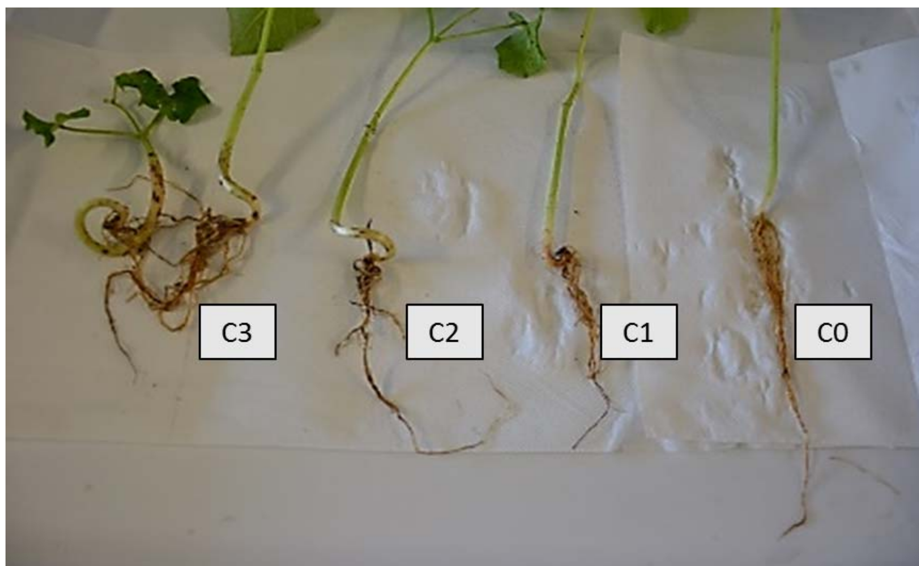

Fig. 3 – Bean plants with typical symptoms of *Rhizoctonia solani* disease and severity disease classes.
Piante di fagiolo con i tipici sintomi di malattia di *Rhizoctonia solani* e classi di gravità di malattia.

Biostimulant activity

The experiment on biostimulant activity on bean plants was carried out with the same products described above and in absence of the pathogen. The effect of treatments were evaluated by measuring leaf area and total dry weight as previously specified.

In vitro assays

To evaluate the ability of microbial compounds to inhibit the colony growth of R. solani, plate tests were performed on PDA. Bok, Sut, T22 and TJ40 were separately tested at 20 g/L, 5 ml/L, 0.6 g/L and 5 × 106 cfu/ml, respectively. Two experiments were carried out following two methods: submerged colony (SC) and well diffusion (WD). In the first a 5 cm diameter plug removed from the edge of the active growing

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colony of Rs was submerged in 2 ml water suspension of each product (Pane et al., 2013). After an overnight incubation at 25 °C, the plug was transferred in the centre of a PDA Petri plate (diameter 90 mm), then incubated at 25 °C for 4 days in the dark. In the second experiment, a modified procedure of well diffusion (WD) assay (Magaldi et al., 2003) was used. Once the agarized medium had solidified, three wells, each 5 mm in diameter, were cut out of the medium equidistantly from the centre from each PDA plate, and 100 µl of the product suspension were placed into each well. A plug (5 mm diameter) removed from the edge of the growing pathogen colony was inoculated in the centre of the plate, and then the plates were incubated at 25 °C for 4 days in the dark. The inhibition of colony pathogen growth by the compounds were indicated as follow: 100 - 80 % (++++), 80 - 50 % (+++), 50 – 30 % (++), < 30 % (+) and not inhibited (-), compared to control. Both experiments were repeated two times with 5 replicates for each treatment.

Statistical analysis

Percentage data were arcsine transformed before statistical analysis and back transformed data are reported in the tables. Data were submitted to one-way analysis of variance (ANOVA univariate, Statgraphic Plus Version 2.1; Statistical Graphics Corp., USA 1996), and the means were compared by using LSD multiple range test ($P < 0.05$).

Results

Growth chamber experiments

No natural infection by Rs or other seed-borne pathogens was observed on seeds examined before seeding.

Tab. 2 - Effect of microbial products on disease incidence and disease severity index (0–100) of bean plants three weeks after *Rhizoctonia solani* inoculation to the substrate at seeding time.
Effetto di prodotti a base di microrganismi sull'incidenza e indice di gravità della malattia (0-100) su piante di fagiolo tre settimane dopo l'inoculazione di *Rhizoctonia solani* nel substrato al momento della semina.

Treatment	Disease incidence (%)	Disease severity index (%)
Infected control	100.0 ± 0.0 c	91.1 ± 6.2 d
T22	97.9 ± 3.6 bc	75.0 ± 2.7 ab
TJ40	97.4 ± 4.4 bc	80.8 ± 1.5 bc
Bok/S	100.0 ± 0.0 c	85.2 ± 5.1 cd
Bok/S + Sut/S +Sut/L	88.6 ± 4.4 ab	69.6 ± 2.6 a
Sut/S + Sut/L	86.2 ± 8.2 a	68.7 ± 4.7 a
Sut/Se + Sut/S + Sut/L	89.3 ± 4.9 ab	68.7 ± 7.4 a

Means (± SD) followed by the same letter in a column are not significantly different according to LSD test ($P < 0.05$).

Data on the effect of treatments on DI and DS of bean plants inoculated with Rs at seeding time are reported in Table 2. The pathogen was highly pathogenic for beans plants as showed by the values of DI (100%) and DS (91.1 ± 6.2 %). The combined treatment Bok/S+Sut/S+Sut/L and the treatments

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Sut/S+Sut/L or Sut/Se+Sut/S+Sut/L significantly ($P < 0.05$) decreased both DI and DS compared with the control. These treatments were more efficient in reducing DS (23.6 % - 24.6 %) than DI (10.7 % - 13.8 %). Trianum (T22) and TJ40 reduced DS by 17.7 % and 11.3 %, respectively, compared to the control, and T22 gave statistically similar DS reduction to Bok/S+Sut/S+Sut/L, Sut/S+Sut/L or Sut/Se+Sut/S+Sut/L.
In Table 3 the effect of treatments on total plant dry weight (DW) and on total leaf area (LA) of bean plants inoculated with Rs at seeding time is reported. Bok/S+Sut/S+Sut/L, Sut/S+Sut/L, Sut/Se+Sut/S+Sut/L and T22 significantly ($P < 0.05$) increased both DW and LA with respect to the control. These treatments increased DW from 30,0 % to 72.5 % and LA from 54.6 to 171.1 %. The highest increases of DW was obtained with Bok/S+Sut/S+Sut/L and Sut/Se+Sut/S+Sut/L (55.0 % and 72.5 %, respectively), and the highest increase of LA was achieved by Bok/S+Sut/S+Sut/L (171.1 % compared to the control). Both DW and LA obtained with T22 treatment did not differ from Sut/S+Sut/L or Sut/Se+Sut/S+Sut/L.

Tab. 3 - Effect of microbial products on dry weight and leaf area of bean plants three weeks after *Rhizoctonia solani* inoculation to the substrate at seeding time.
Effetto di prodotti a base di microrganismi sul peso secco e l'area fogliare di piante di fagiolo tre settimane dopo l'inoculazione di *Rhizoctonia solani* nel substrato al momento della semina.

Treatment	Total plant[a] dry weight (mg)	Total leaf area (mm^2)
Infected control	4.0 ± 1.0 a	644.2 ± 160.5 a
T22	5.9 ± 0.5 bc	1090.0 ± 265.0 b
TJ40	4.6 ± 0.8 a	775.9 ± 1910.8 ab
Bok/S	4.4 ± 0.8 a	795.5 ± 126.2 ab
Bok/S + Sut/S +Sut/L	6.2 ± 0.8 c	1746.8 ± 367.4 c
Sut/S + Sut/L	5.2 ± 0.4 b	1059.0 ± 107.7 b
Sut/Se + Sut/S + Sut/L	6.9 ± 0.4 c	995.7 ± 103.9 b

[a] plants were three weeks old.
Means (± SD) followed by the same letter in a column are not significantly different according to LSD test ($P < 0.05$).

The effect of treatments on DI and DS of bean plants inoculated with Rs at seedling stage time is reported in Table 4. The pathogen caused very high DI (100 %) and 74.5 ± 2.1 % of DS. All treatments, except Bok/S, significantly ($P < 0.05$) reduced values of both disease parameters. The DI was reduced from 25.3 % (TJ40) to 41.4 % (Bok/S+Sut/S+Sut/L). The DS was reduced from 20.3 % (TJ40) to 38.7 % (Bok/S+Sut/S+Sut/L). Treatment with Bok/S+Sut/S+Sut/L and Sut/S+Sut/L showed the lowest DS values, 45.7 ± 7.7 % and 48.6 ± 9.0 % respectively, compared to 74.5 ± 2.1 % of control.
In Table 5, the effect of treatments on total plant dry weight (DW) and on total leaf area (LA) of bean plants inoculated with Rs at seedling emergence is reported. All treatments, except Bok/S, significantly ($P < 0.05$) increased both DW and LA values. The highest increase of DW with respect to the infected control was obtained with Sut/Se+Sut/S+Sut/L (98.1 %). With the exception of Bok/S, values of LA showed by all treatments were significantly ($P < 0.05$) similar. These effective treatments increased LA from 21.9 % (T22) to 29.3 % (Bok/S+Sut/S+Sut/L) with respect to the control.

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Tab. 4 - Effect of microbial products on disease incidence and disease severity index (0–100) of bean plants three weeks after *Rhizoctonia solani* inoculation to substrate at seedling stage time.
Effetto di prodotti a base di microrganismi sull'incidenza e indice di gravità della malattia (0-100) su piante di fagiolo tre settimane dopo l'inoculazione di *Rhizoctonia solani* nel substrato allo stadio di piantina.

Treatment	Disease incidence (%)	Disease severity index (%)
Infected control	100.0 ± 0.0 b	74.5 ± 2.1 d
T22	63.9 ± 6.2 a	53.3 ± 2.9 ab
TJ40	74.7 ± 4.3 a	59.4 ± 5.0 bc
Bok/S	93.3 ± 11.5 b	65.2 ± 3.1 cd
Bok/S + Sut/S +Sut/L	58.6 ± 15.8 a	45.7 ± 7.7 a
Sut/S + Sut/L	60.0 ± 11.9 a	48.6 ± 9.0 a
Sut/Se + Sut/S + Sut/L	70.4 ± 2.6 a	54.2 ± 2.1 ab

Means (± SD) followed by the same letter in a column are not significantly different according to LSD test ($P < 0.05$).

Tab. 5 - Effect of microbial products on total dry weight and leaf area of bean plants three weeks after *Rhizoctonia solani* inoculation to substrate at seedling stage time.
Effetto di prodotti a base di microrganismi sul peso secco e l'area fogliare di piante di fagiolo tre settimane dopo l'inoculazione di *Rhizoctonia solani* nel substrato allo stadio di piantina.

Treatment	Total plant[a] dry weight (mg)	Total leaf area (mm^2)
Infected control	5.2 ± 0.6 a	785.8 ± 87.8 a
T22	8.7 ± 0.8 c	958.3 ± 43.5 bc
TJ40	7.1 ± 0.9 bc	980.8 ± 43.2 c
Bok/S	5.2 ± 0.9 a	793.3 ± 87.8 a
Bok/S + Sut/S +Sut/L	6.9 ± 1.1 b	1015.8 ± 87.8 c
Sut/S + Sut/L	7.9 ± 1.4 bc	947.5 ± 33.2 bc
Sut/Se + Sut/S + Sut/L	10.3 ± 0.9 d	977.5 ± 57.5 c

[a] plants were four weeks old.
Means (± SD) followed by the same letter in a column are not significantly different according to LSD test ($P < 0.05$).

Tab. 6 - Biostimulant effect of the microbial products on four weeks-old bean plants.
Effetto biostimolante di prodotti a base di microrganismi su piante di fagiolo di quattro settimane.

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Treatment	Total plant dry weight (mg)	Total leaf area (mm^2)
Untreated control	4.8 ± 0.8 a	732.4 ± 57.0 a
T22	6.6 ± 1.3 cd	1155.6 ± 292.2 bc
TJ40	5.1 ± 0.3 ab	899.0 ± 132.6 ab
Bok/S	5.7 ± 0.6 abc	1254.6 ± 328.3 bc
Bok/S + Sut/S +Sut/L	6.6 ± 0.2 cd	1669.4 ± 223.2 d
Sut/S + Sut/L	6.3 ± 0.7 bc	1267.5 ± 253.8 bc
Sut/Se + Sut/S + Sut/L	7.7 ± 1.1 d	1309.3 ± 114.6 cd

Means (± SD) followed by the same letter in a column are not significantly different according to LSD test ($P < 0.05$).

Table 6 reports data on the biostimulant effect of the treatments on DW and LA. Four treatments, T22, Bok/S+Sut/S+Sut/L, Sut/S+Sut/L and Sut/Se+Sut/S+Sut/L showed DW values significantly ($P < 0.05$) higher than the untreated control. The increase obtained with these treatments varied in a range from 31.2 % (Sut/S + Sut/L) to 60.4 % (Sut/Se+Sut/S+Sut/L). Leaf area was significantly ($P < 0.05$) enhanced by almost all treatments, except for TJ40. The treatment that showed the highest LA value was Bok/S+Sut/S+Sut/L that increased LA by 127.9 %.

In vitro assays

Almost all products showed to reduce Rs growth in both experiments carried out with different methods (Tab. 7). *Trichoderma* T22 completely inhibited the pathogen growth in SC and WD experiments (Fig. 4). *Trichoderma* TJ40 inhibited Rs growth in a range of 80-50 % in SD assay), and 50-30 % in WD assay. Among EM products, Bok reduced pathogen growth less than 30 % in both assays, while Sut slightly reduced Rs growth in WD assay only.

Tab. 7 - Effect of treatments on mycelial growth of *Rhizoctonia solani* in the two *in vitro* assay.
Effetto di trattamenti con prodotti a base di microrganismi sulla crescita miceliare di *Rhizoctonia solani* nei due saggi *in vitro*.

Treatment	Submerged colony	Well diffusion
Control	-	-
T22	++++	++++
TJ40	+++	++
Bok	+	+
Sut	-	+

Inhibition range: 80-100 % (++++), 80 - 50 % (+++), 50 – 30 % (++), < 30 % (+) significant reduction compared with control. Not inhibited (-).

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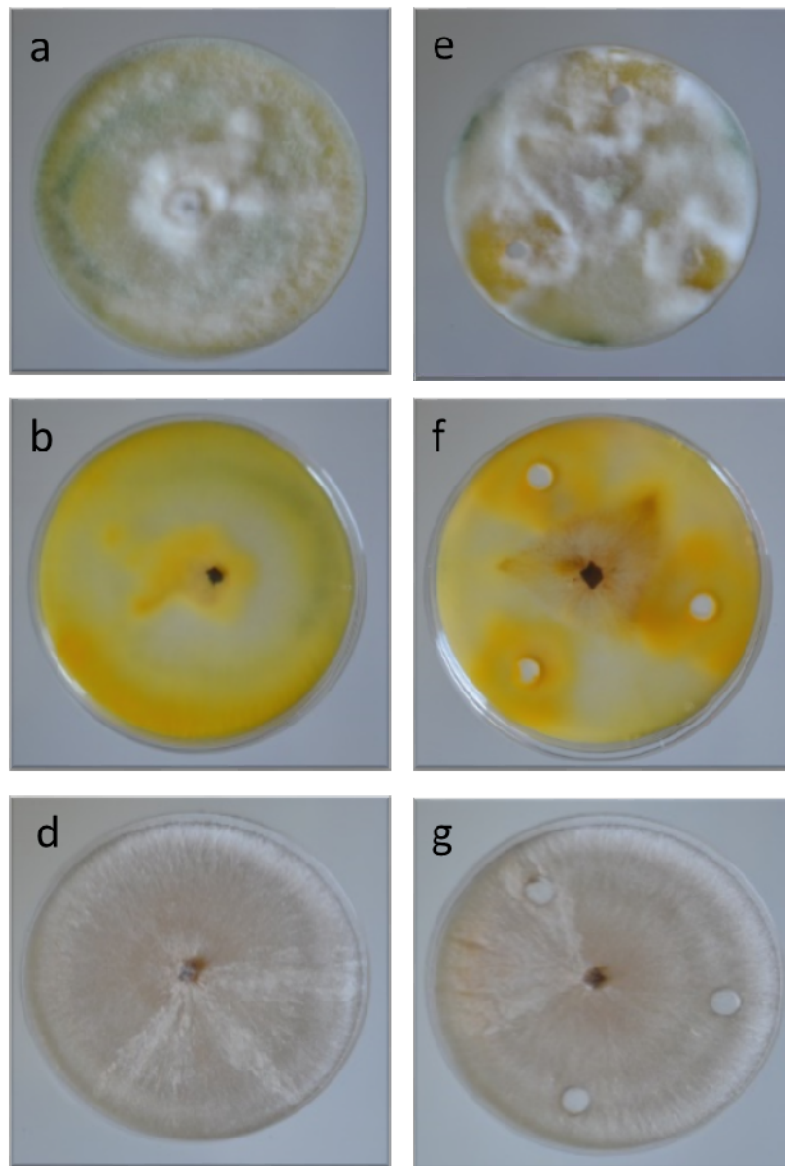

Fig. 4 - Effect of T22 on mycelial growth of *Rhizoctonia solani* after 7 days of incubation on PDA in the submerged colony assay (a-d) and in well diffusion assay (e-f), control plates (d, g). Top (a, d, e, g) and bottom view of plates (b, f).
Effetto di T22 sulla crescita miceliare di *Rhizoctonia solani* dopo 7 giorni d'incubazione su PDA nel saggio "submerged colony" (a-d) e nel saggio "well diffusion" (e-f); piastre controllo (d, g); lato superiore (a, d, e, g) e lato inferiore (b, f) delle piastre.

Conclusions

Rhizoctonia solani is a significant threat to global food security and agro-forestry industries (Hane et al., 2014). Chemical control methods may not be feasible nor economical for the control of many soil-borne pathogens including *R. solani* (Paulitz, 2006). Agronomic methods, such as crop rotation are not always effective, since many crops are susceptible to this pathogen. Moreover, conventional breeding strategies are scarcely employed, since only low or moderate levels of genetic resistance have been found (Bradley et al., 2001; Kluth & Varrelmann, 2010). Microbial compounds based on antagonistic microorganisms represent an environmental friendly method to control numerous soil-borne diseases on various crops

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(Harman, 2000; Haas & Defago, 2005). Antagonistic microorganisms are components of specific plant protection products; however, microorganisms, which can potentially act as antagonists, can be contained in plant biostimulant compounds. European agricultural and food safety policies have integrated environmental considerations and are promoting the safe use of agricultural inputs. This applies to alternative solutions including the use of plant biostimulants and biopesticides that are used under the integrated crop management schemes (Report for the European Commission, 2014).
As far as we know, no scientific studies have compared the effect of antagonistic microorganisms contained in specific products with that of plant biostimulant compounds in the control of soil-borne disease. The present study showed that *R. solani* disease of bean can be significantly reduced both by antagonistic microorganisms and by compounds which their primary function is the promotion of plant growth. Our findings were obtained in high inoculation pressure disease that has been much greater than that found in typical greenhouse conditions or in the field. Even in these conditions, the products showed various levels of efficacy.
Under our experimental condition, the EM-5 Sutociu treatment alone or combined with EM Bokashi 2-fi, applied as recommended by manufacturer, showed almost always the same disease control as the single treatment with Trianum-P (*Trichoderma harzianum* T22), which is a well-known biocontrol agent. EM Bokashi 2-fi applied singly and only once to substrate, did not reduced the disease. Considering that EM-5 Sutociu contains the same microorganisms in the same concentrations of EM Bokashi 2-fi, we can explain this result by assuming that they are differently formulated; EM-5 Sutociu contains microorganisms suspended in substances such as grappa and allium extract, and EM Bokashi 2-fi is based on bran enriched with microorganisms. The treatment TJ40 applied once to substrate was as effective as T22 in reducing disease severity. The efficacy of TJ40 in reducing *R. solani* disease confirmed what demonstrated in in vitro assay by Leonardi et al., (2014).
The biostimulant product EM-5 Sutociu, the biofungicide T22 and *Trichoderma* sp. J40 probably reduced the disease by different modes of action, as demonstrated by the in vitro assays. In fact, as expected, *T. harzianum* T22 and *Trichoderma* sp. J40, even if at different levels, were able to reduce *R. solani* colony growth more actively then EM-5 Sutociu. Hence, this biostimulant exerted a disease control probably mainly by the enhancement of plant defence responses and by very slightly antagonism towards the pathogen. This is in accordance with Calvo et al. (2014) who stated by that biostimulants are not pesticides, because they do not act directly against pathogens, being the plant growth promotion their primary function. Veronesi et al., (2009), have also showed the involvement of plant defence mechanisms against biotic stresses.
The biostimulant effect of EM-5 Sutociu and EM Bokashi 2-fi was confirmed also in the case of beans; in particular, they both increased the leaf area. Trianum-P also showed a biofertilization effect, consistently to what demonstrated by other authors (Windham et al., 1986; Harman et al., 2004, Matsouri et al., 2010).
In conclusion, this study shows that the application of biostimulant products, in particular EM-5 Sutociu, as well the biocontrol agent T22, could simultaneously increase bean plant growth and reduce *R. solani* disease, although further studies are necessary to verify these effects under different management practices.

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