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Short Communication: The potential of various indigenous *Trichoderma* spp. to suppress *Plasmodiophora brassicae* the pathogen of clubroot disease on cabbage

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Abstract. *Suada IK. 2017. Short Communication: The potential of various indigenous* Trichoderma *spp. to suppress* Plasmodiophora brassicae*, the pathogen of clubroot disease on cabbage. Biodiversitas 18: 1424-1429.* On the island of Bali, cabbage (*Brassica oleracea* L.) is a vegetable that has high economic value especially for farmers who cultivate it as a cash crop. However, the clubroot disease caused by the microbial pathogen *Plasmodiophora brassicae* decreases its productivity. Much effort has been expended in attempts to decrease the pathogen attack by use of synthetic fungicides, however, this has not had much success; more over such pesticide applications generate environmental pollution. Therefore, biological control using *Trichoderma* or other organisms antagonistic to the pathogen is an attractive alternative. The purpose of this study was to find, isolate and identify *Trichoderma* spp. able to decrease the disease incidence and increase plant growth. Twelve indigenous *Trichoderma* isolates were tested on cabbage plants grown in polybags containing local soil media culture treated with the *Trichoderma* inocula at a concentration of $1.5x10^6$ CFU per polybag. The results showed that three *Trichoderma* isolates, i.e. *T. hamatum*-1*, T. harzianum*-1, and *T. harzianum*-2, were able to decrease the clubroot disease and increase cabbage growth as well.

Keywords: Biocontrol, cabbage, clubroot, *Trichoderma*

INTRODUCTION

Cabbage (*Brassica oleracea* L.) is a popular vegetable beneficial to health because it contains vitamins A, B1, C, and minerals, and is therefore in high demand on the island of Bali, Indonesia. However, the production of local cabbages in Bali has been in steady decline since 2010: in that year, the production of cabbages in Bali amounted to 47,077 tons, in 2011 it was 42,926 tons, in 2012 40,167 tons, but in 2013 it only reached 35,781 tons (Central Bureau of Statistics of Bali Province 2014). This decline in production was closely related to the observed high incidence of clubroot disease caused by the microbial pathogen *Plasmodiophora brassicae* Woronin, responsible not only for the disease in cabbage plants, but also for attacking other members of the family Brassicaceae.

According to local farmers, clubroot disease is an important issue in every crop season and occurs in almost every cabbage-growing area in Bali. Disease control using pesticides is often not effective against soil borne pathogens such as the clubroot pathogen. Moreover, excessive use of fungicides is polluting the environment. As a result, public attention has been increasingly drawn to environmental safety concerns, leading to limitations being placed on pesticide application.

A potential alternative to the use of chemical pesticides in the control of clubroot disease of cabbages is an environmentally friendly control system based on biological agents such as fungi or bacteria antagonistic to the clubroot pathogen. One such antagonist with potential to be used as a biocontrol agent is the fungal genus *Trichoderma* Persoon. Besides being able to protect the cabbage against clubroot-disease caused by *Plasmodiophora brassicae*, *Trichoderma* also can control various other diseases caused by fungal pathogens (Cheah and Page 1997; Navi and Bandyopadhyay 2002). Fungal pathogens that have been reported as being controlled to some degree by *Trichoderma* spp. Include *Pythium, Phytophthora, Fusarium, Rhizoctonia, Sclerotium,* and *Verticillium* (Nederhoff 2001; Agrios 2005; Arya and Perello 2010). The use of introduced *Trichoderma* spp. has been reported to reduce pathogen attacks by about 25%, while the use of fermented livestock manure combined with *Trichoderma* spp. in cabbage reduced pathogen attack by 51% (Legowo 2010). In addition, to acting as antagonists against pathogens, *Trichoderma* can also act as a plant growth promoter, because the interaction between *Trichoderma* and plants has been reported to stimulate formation of plant growth hormones(Harman et al. 1991).

In the research literature, there are documented cases of obstacles encountered in testing for the effectiveness of various antagonist agents against pathogens. The major factors that influence the effectiveness of antagonist agents are environmental extremes include temperature fluctuations, ultraviolet light, pH, availability of water and nutrients, as well as competition from indigenous microflora (Agrios 2005; Renwick and Poole 1989; Zhu et al. 2016). As a result, there are problems in the adaptation of some introduced biological agents to the environment of their application site.

Microbial control agents introduced to help protect crops against pathogens that cause root disease must adapt to the environment of the application site and to the rhizosphere of the crop plants if they are to function properly as antagonist agents. Moreover, an antagonist agent is usually propagated in an artificial media so becoming accustomed to the optimum conditions of that medium. This tends to reduce its completive abilities when introduced to the open field conditions of a growing crop. Furthermore, the genetics of the pathogenic microbe and also of the antagonistic agent generally has a capacity to change quickly, and so the effectiveness of the antagonist against the pathogen can change rapidly as well. At the site of introduction, biological agents can be inhibited by the new environmental conditions resulting in lower than expected effectiveness under field conditions.

Weller (1988), in his wheat research, has clearly revealed that although introduced microbes may dominate in a new location for the few first weeks, by the end of the growing season they can decline in vigor and their population drop to less than 2% of the original population. Even genetically modified agents can experience the same thing, such that their population shrinks drastically and they no longer remain effective as biological agents.

The research reported in this paper was undertaken to obtain and identify local *Trichoderma* spp. isolates able to be used as biocontrol agents in cabbage crops grown in Bali. The purpose of the study was to culture these *Trichoderma* isolates and apply them at populations suitable for controlling clubroot disease and enhancing growth of cabbage plants under local conditions.

MATERIALS AND METHODS

Soil sampling

Soils were sampled in farmers' vegetable gardens, from the rhizosphere of various type of cabbages infected by clubroot disease in the area of Bedugul, Tabanan, Bali. Each sample of 200 g was placed in a plastic bag. The samples were packaged in a box that had been filled with ice to protect them from sun damage and overheating, and then transported to the Biotechnology Laboratory of the Faculty of Agriculture, University of Udayana, Denpasar, Bali, Indonesia.

Isolation and identification of *Trichoderma* spp.

In the Biotechnology Laboratory, isolation of *Trichoderma* spp. from the soil samples was carried out by culturing on PDA (Potato Dextrose Agar) media containing100 ppm Streptomycin as an antibiotic for bacteria.

Colonies of growing fungi were subcultured and then identified by observation under a microscope; matching the fruiting bodies of the fungal isolates to reference images documented in Alexopoulos and Mims (1979), Barnett and Hunter (1972), CMI (1988), Webster and Weber (2007), Domsch et al. (1980), and Watanabe (2002), and also by using the taxonomic key of Samuels et al. (2006). The right *Trichoderma* inocula were subcultured using a single spore isolation technique. A serial dilution method was used to obtain dilutions of 10^{-1}-10^{-7}. One milliliter of suspension of dilution rate of 10^{-3}-10^{-7} was cultured in sterile PDA medium containing 100 ppm Streptomycin to prevent bacterial contaminants growth.

Design of the experiment to test *Trichoderma* isolates on cabbage plants

The experiment to test isolates was performed in a greenhouse of the Faculty of Agriculture. Polybags (diameter 12.5 cm x height 20 cm) received 2.5 kg of a mixed medium (local soil:compost, at a ratio of 3:1). Each polybag was planted with one three-week-old cabbage seedlings. For every isolate to be tested, 9 polybags were assigned. Altogether, twelve *Trichoderma* isolates were tested in the experiment, and their impacts on cabbage plant growth was compared with a control treatment that consisted of cabbage plants with no *Trichoderma* application. Thus, the experiment consisted of a total of thirteen treatments with three replications per treatment (each with three sample plants per polybag), arranged in a completely randomized design. Each of the twelve *Trichoderma* treatments received $1.5x10^6$ spores of the particular assigned *Trichoderma* isolate in 200 ml water, applied as a drench to the soil around the base of the cabbage plants.

Maintenance of the cabbage plants

The polybags were fertilized twice; i.e. at one and three weeks after planting. Fertilizer was given at a dose of 1.3 g/plant ZA (*Zwavelzure Ammoniak*, 20.8% Nitrogen), 1.8 g/plants TSP (Triple Super Phosphate, 45% P_2O_5), and 0,7 g/plant KCl (Potassium chloride, 60% K_2O). Plants were watered until the soil moisture was stable at field capacity. Plants of all treatments were sprayed with foliar fertilizer DI Grow (liquid organic fertilizer contained macro, micro elements, and growth regulator) at a concentration of 2 cc/L at a dose equivalent to 400 L/ha.

Observation variables

The observed variables were: plant height, measured from the ground to the tip of the leaf held erect; total number of leaves; number of galls (i.e. number of large and/or small galls per plant), counted after the plants were upended at the end of the experiment; percentage of plants attacked (%), calculated from the number of plants that had a gall after the plant was upended; leaf chlorophyll content, measured by a SPAD Chlorophyll Meter; leaf area, measured by a Leaf Area Meter. All these variables were observed at7 weeks after planting, i.e. prior to the plants forming cabbage hearts.

Data analysis

Data were analyzed by ANOVA appropriate to a completely randomized design (CRD), followed by a Duncan's Multiple Range Test at a 1% and 5% significance level, to differentiate the effects of isolates.

RESULTS AND DISCUSSION

Antagonistic *Trichoderma* spp.

From the soil obtained in the rhizosphere of various plants sampled at the study site, twelve specimens of *Trichoderma* spp. were isolated in the laboratory. The characteristics of the twelve isolates are described in Table 1 and Figure 1.

Based on observation by microscope, the isolates could be assigned to four different groups with the characteristics shown in Table 2.

As listed in Table 2, the twelve isolates were identified as belonging to four *Trichoderma* spp., namely *T. hamantum, T. harzianum, T. polysporum,* and *T. koningii*. Generally, the *Trichoderma* fungal colonies were found to be white-green in color after three days growth on PDA but they gradually turned bluish toward the seventh days. Within 6 to 7 days, colonies had reached a diameter of 9 cm, which resulted in them reaching the outer edge of the media in the petri dishes.

Performance of the cabbage plants in the experiment evaluating the *Trichoderma* isolates

Observations carried out on the cabbage plants evaluated for the effects of the *Trichoderma* isolates comprised measurements of plant height, leaf number, leaf area, and leaf chlorophyll at seven weeks after planting, just before the plants began to form cabbage hearts.

Plant height and total leaf number did not show a significant difference between the isolate treatments (based on Duncan Multiple Range test at 5% level). However, there was a significant difference between isolate treatments in leaf area and leaf chlorophyll. The largest leaf area was found in isolate treatment Tri-1 (*T. hamatum*-1) with a total leaf area of 95.24 cm^2 leaf area. Its chlorophyll estimate was also the highest, namely 3596.26 SPAD units of chlorophyll (see Wood et al. 1992). These results indicated a beneficial response of the cabbage plant to the fungus *T. hamatum*-1, an interaction that promoted plant growth (Table 3). According to Suwahyono and Wahyudi (2004), *Trichoderma* can act not only as a biological control agent, but the fungus can also act as a driver of growth, improving plant performance: *Trichoderma* can stimulate the formation of growth hormones that enhance plant growth. *Trichoderma* species that have been reported to act in this way are *T. harzianum, T. polysporum*, and *T. viridae*. In this current study, the most probable isolate with the described property was *T. hamatum*-1. Compared to other isolates, *T. hamatum* in this study was better in its effects on cabbage plant performance than *T. harzianum* and *T. polysporum.*

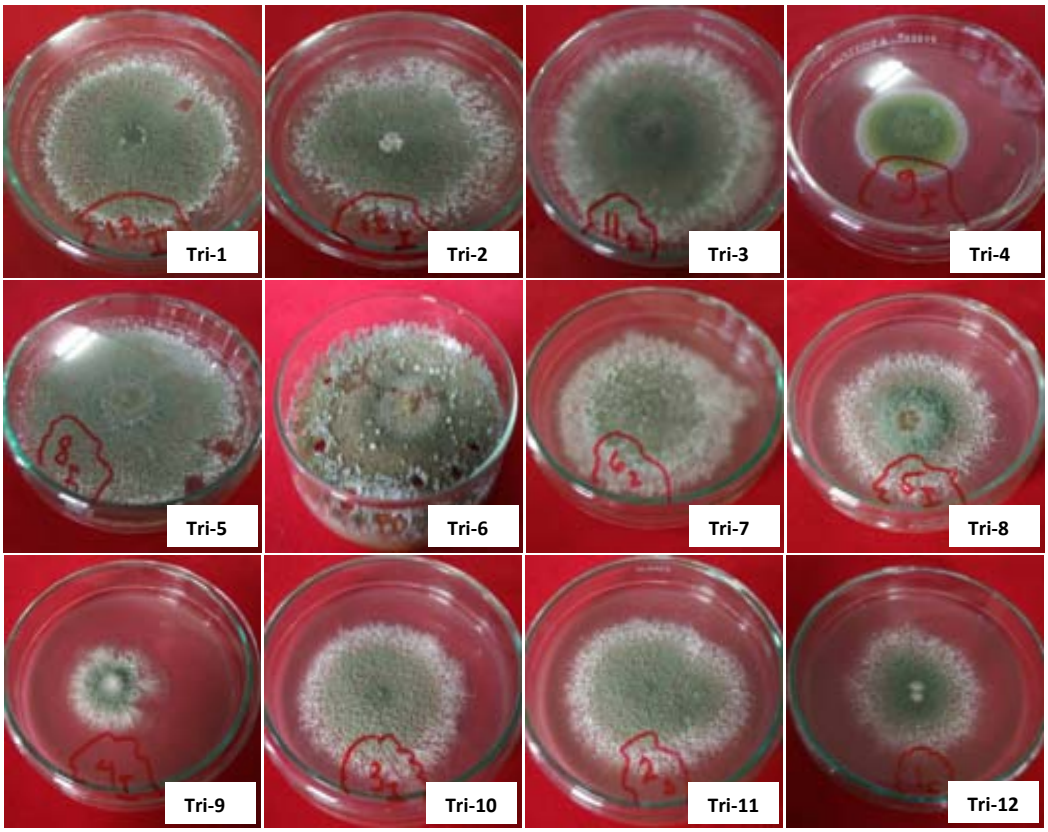

Figure 1. Various types of *Trichoderma* spp. colonies isolated from the rhizosphere of healthy plants in farmers' fields where clubroot disease was prevalent

Table 1. Characteristics of the colony development of twelve *Trichoderma* spp. isolates cultured on PDA medium

Isolate	Color at colony age (day 1 to 7)							Colony form
	1	2	3	4	5	6	7	
Tri-1	White	Greenish white	Greenish white	Greenish white old	Light green	Green	Dark green	Round
Tri-2	White	Greenish white	Light green	Greenish white old	Light green	Green	Green	Round
Tri-3	White	White	Greenish white	Greenish white old	Light green	Green	Dark green	Round
Tri-4	White	White	Green	Dark green	Light green	Dark green	Dark green	Round
Tri-5	White	Greenish white	Greenish white	Greenish white old	Light green	Green	Dark green	Round
Tri-6	White	Greenish white	Greenish white	Greenish white old	Light green	Green	Dark green	Round
Tri-7	White	Greenish white	Light green	Greenish white old	Light green	Green	Dark green	Round
Tri-8	White	Greenish white	Light green	Greenish white old	Light green	Green	Dark green	Round
Tri-9	White	Greenish white	Light green	Greenish white old	Light green	Green	Dark green	Round
Tri-10	White	White	Greenish white	Greenish white old	Light green	Green	Dark green	Round
Tri-11	White	Greenish white	Greenish white	Greenish white old	Light green	Green	Dark green	Round
Tri-12	White	Greenish white	Light green	Greenish white old	Light green	Green	Dark green	Round

Note: Tri=*Trichoderma* spp. Numbers that follow Tri is an identifying numbers for isolates obtained from various healthy plants in farmers' fields where clubroot disease was endemic

Table 2. Characteristics of *Trichoderma* spp. isolated from the rhizosphere of various healthy plants

Isolate	Microscopic characteristics			Species
	Conidiophore	Phialides	Conidia	
Tri-1	Erect, branched	Short, thick	Oval	*T. hamantum*-1
Tri-2	Erect, branched	Short, thick	Oval	*T. harzianum*-1
Tri-3	Branched	Long, very thick	Oval	*T. polysporum*-1
Tri-4	Erect, branched	Short, thicker	Oval	*T. harzianum*-2
Tri-5	Erect, branched	Short, thick	Oval	*T. harzianum*-3
Tri-6	Erect, branched	Short, thick	Oval	*T. hamantum*-2
Tri-7	Erect, branched	Short, thick	Oval	*T. hamantum*-3
Tri-8	Erect, branched	Small, taper	Oval and round	*T. koningii*-1
Tri-9	Erect, branched	Short, thick	Oval	*T. hamantum*-4
Tri-10	Branched	Long, very thick	Oval	*T. polysporum*-2
Tri-11	Branched	Long, very thick	Oval	*T. polysporum*-3
Tri-12	Erect, branched	Small, taper	Oval and round	*T. koningii*-2

Note: Tri=*Trichoderma* spp. Numbers that follow Tri are identifying numbers for the isolates obtained from various healthy plants in the field areas where clubroot disease was prevalent.

Table 3. Effect of isolates of *Trichoderma* spp. on several cabbage plant variables at seven weeks after planting

Treatment	Variables of plant performance			
	Height (cm)	Total leaf number	Leaf area (cm^2)	Leaf chlorophyll (SPAD)
Control (no Tri)	14.45±3.23 a	9.33±0.89 a	28.43±43 b	1007.84±2.56 d
Tri-1	17.79±2.44 a	11.00±5.14 a	95.24±6.75 a	3596.26±3.66 a
Tri-2	16.45±4.35 a	10.00±2.11 a	64.36±3.76 ab	2413.50±7.54 b
Tri-3	14.88±1.57 a	9.66±3.55 a	43.42±5.22 b	1577.44±6.77 c
Tri-4	16.08±2.65 a	9.66±2.45 a	64.01±6.32 ab	2349.16±12.69 b
Tri-5	14.56±3.56 a	9.33±1.79 a	31.66±4.33 b	1122.34±11.54 d
Tri-6	14.45±3.77 a	10.00±2.19 a	35.48±3.22 b	1321.98±32.56 d
Tri-7	14.54±1.33 a	9.33±2.17 a	49.09±2.11 ab	1819.27±18.23 c
Tri-8	17.06±2.35 a	10.33±1.35 a	58.40±3.24 ab	2219.93±10.23 b
Tri-9	16.54±2.33 a	9.66±3.44 a	61.45±3.44 ab	2284.09±9.34 b
Tri-10	15.54±1.14 a	10.00±1.35 a	62.25±4.68 ab	2311.34±16.88 b
Tri-11	15.63±4.12 a	9.66±2.33 a	41.38±3.56 b	1596.02±24.15 c
Tri-12	14.94±3.66 a	10.00±2.56 a	26.09±2.77 b	965.33±13.24 d

Note: Tri=*Trichoderma* spp. Numbers that follow Tri are numbers identifying particular isolates obtained from various healthy plants in a field area where clubroot disease was prevalent. Data are expressed as means±SDs. Values followed by the same letters in the same column do not differ significantly at 5% probability level according to Duncan Multiple Range test

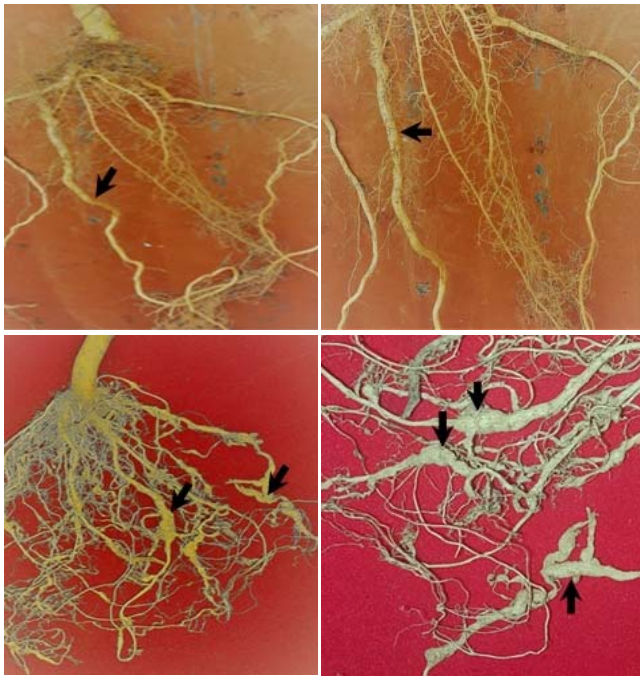

Figure 2. The appearance of the plant roots at seven weeks after planting. Asymptomatic roots (*above*) and galled roots (*below*)

Table 4. Incidence of clubroot disease observed in cabbage plants treated with different *Trichoderma* spp. isolates

Treatment	Observation variables	
	Gall number (pieces/plant)	Disease incidence (%)
Control (no Tri)	8.94±3.03 a	100.00 a
Tri-1	0.00 d	0.00 d
Tri-2	0.00 d	0.00 d
Tri-3	8.22±2.00 a	100.00 a
Tri-4	2.33±0.21 c	11.11±0.25 c
Tri-5	7.89±2.13 a	55.55±05.00 b
Tri-6	7.45±2.22 a	55.55±1.00 b
Tri-7	7.35±3.02 a	33.33±3.95 b
Tri-8	4.33±2.41 b	11.11±2.13 c
Tri-9	2.78±0.02 c	11.11±2.16 c
Tri-10	7.35±1.80 a	55.55±05.00 b
Tri-11	7.78±2.02 a	44.44±3.55 b

Note: Tri=*Trichoderma* spp. Numbers that follow Tri are numbers identifying particular isolates obtained from various healthy plant in a field area where clubroot disease was prevalent. Data are means±SDs. Values followed by the same letters within the same column are not significantly different at a 5% level of significance, based on Duncan Multiple Range test. Percentage data were analyzed after arc-sin $\sqrt{(x+1/2)}$ conversion.

Incidence of clubroot on cabbage plants

The effect of the *Trichoderma* isolates on incidence of clubroot in the cabbage was rated based on two variables i.e. the number of clubroot galls and the percentage of infected plants (Figure 2). It proved not possible to effectively categorize all galls according to size into large or small, and so all galls irrespective of size were counted as single galls. The percentage of infected plants was obtained by dividing the number of plants with gall(s) by the total number of plants observed, expressed as a percentage.

Tri-1, that is *T. hamatum*-1, completely suppressed the formation of galls, so that the incidence of clubroot in this treatment was zero, this suggests that Tri-1 may be antagonistic to the growth of the pathogen (*P. brassicae*). Furthermore, Tri-2 (*T. harzianum*-1) also demonstrated the same total suppression of gall formation, while Tri-4, Tri-8 and Tri-9 showed a relatively low disease incidence (11.11%) (Table 4). The isolates Tri-1 and Tri-2 were effective in disease-suppression and also appeared to promote the health of the cabbage plants as indicated by high total chlorophyll estimates of 3596.26 and 2413.50 SPAD respectively. That means that the two *Trichoderma* isolates may act not only as biological control agents, but may also enhance plant growth. Plants with a high leaf chlorophyll content would be expected to have enhanced photosynthesis and hence enhanced ability to form carbohydrates. Many studies have reported that increased chlorophyll concentrations in plant leaves, achieved by improved nutrition, are correlated with higher yields in agricultural and horticultural crops (Wood et al. 1992; Kararurt et al. 2009; Fan et al. 2014).

It is possible that a beneficial *Trichoderma*-crop association results in growth enhancement of the plants via promotion of photosynthesis as indicated by the high chlorophyll content and leaf area observed in Tri-1 and Tr-2 treatments. A conclusion that follows from this research is that *Trichoderma* isolates capable of suppressing clubroot disease in cabbage plants also improved some parameters of cabbage plant growth. The best isolates were *Trichoderma hamatum*-1, followed by *Trichoderma harzianum*-1, and *Trichoderma harzianum*-2. Further research is needed to determine the most effective formulations that can be applied in the field.

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