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# BIOCONTROL AND GROWTH ENHANCEMENT POTENTIAL OF *TRICHODERMA* SPP. ON BROAD LEAF MUSTARD

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## ABSTRACT
Microbial isolates from plant associated habitats are being considered as valid alternatives to synthetic pesticides. The aim of the study was to select beneficial fungi belonging to *Trichoderma* genus, to be added as soil inoculants, in order to develop an innovative, economic and suitable alternative to synthetic fertilizers for growth promotion of plants. The isolated *Trichoderma* were characterized and identified by morphological and microscopic analysis. The antagonistic effects of these *Trichoderma* isolates were tested against three pathogenic fungi; *Sclerotinia minor*, *Fusarium oxysporum* and *Fusarium solani*. Dual culture technique was employed and percentage of inhibition (I %) on mycelial growth of pathogenic fungi was calculated. Isolates BC and KB, showed the highest antagonistic effect against *S. minor* by 100%. Isolate DH and TH showed partial suppression of *F. solani y* 64.74% and 70.94% respectively. Isolates HA and Y restrain *F. oxysporum* by 85.12% and 85.90% respectively. Hence, BC, KB, DH, TH, HA, and Y could be a potential bio-control agent, BCA. In vitro and in vivogrowth promotion study was carried out by seed treatment method.Isolate EO revealed highest growth promotion activity in all parameters. In vitro studies of seedling assay indicated that isolate EO exhibited best effect on almost all the parameters; root length, shoot length, plant wet weight, plant dry weight, germination percentage and seedling vigour index in in vitro condition. However, greenhouse studies indicated that GS showed highest shoot length, TH promoted highest root length and HA showed maximum number of leaves. Significant difference was observed inroot length in greenhouse experiment and plant wet weight in seedling assay at $p \leq 0.05$. The results presented in this study further reinforce the concept of biological control and plant growth promotion by *Trichoderma* as an alternative disease control strategy.

**Keywords**: *Trichoderma spp,* Seed germination, Plant growth promotion, *Sclerotinia minor*, *Fusarium solani, Fusarium oxysoprum*

## INTRODUCTION

Plant diseases substantially reduce quality and quantity of agricultural products.Public concerns over excessive use of agrochemicals are now being perceived by risk on natural resource declination, environmental problems and diminishing returns in intensive agricultural areas. At present, microbial isolates from plant associated habitats are being considered as valid alternatives to synthetic pesticides. Microbial agents include bacteria, fungi and viruses that secrete secondary metabolites to provide growth hormones and enhance transfer of minerals to rhizosphere and induce localized or systemic resistance responses. Based on these beneficial plant–microbe interactions, *Trichoderma* are considered as model organisms to demonstrate its influence on plant health [1].

*Trichoderma* spp. are soil borne saprophytic fungi specially found in temperate and tropical soil, forest humus layer, decomposing organic matter and agricultural soils. Theyare considered as inexpensive biocontrol agent that lives in harmony with beneficial micro-organisms protecting plants against

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pathogen and has moderate effect on soil balance [2]. The ability of *Trichoderma* spp. to produce antibiotics and plant growth regulators help plants to combat pathogens and induce localized or systemic resistance responses [3]. Phytopathological societies and pesticides companies in Nepal have initiated for research and development, field based trials and commercial production, for domestic market using *Trichoderma* species.In Nepal, effective use of *Trichoderma* has been observed in vegetables such as ginger, potato, tomato, cabbage and broad leaf mustard to increase their yield and as biocontrol agents in various organic farms [4, 5, 6, 7].

Broad leaved mustard (*Brassica juncea var. Marpha*) is the most widely cultivated leafy vegetable in Nepal. It is a winter crop from Terai to the mid hills where as it is a summer crop for the high hills. It is rich in Vitamin A, B, C, E, iron, calcium and protein. Fungal pathogens*S. minor* and *S. sclerotium*, has limited production of broadleaf mustard thusincreasinguse of chemical fertilizers for its growth.

In this line, approach attempted in this study was the assessment of the *in vitro* biocontrol potential and plant growth promotion action in in-vitro and in-vivo condition associated with broad leaved mustard.

## MATERIALS AND METHODS

### Fungal isolation

Ten isolates of *Trichoderma* and plant pathogens *S. minor, F. solani* and *F. oxysporium* were used in this study. The *Trichoderma* isolates were obtained from soil of different organic farms and botanical gardens using serial dilution method[8]. Pathogen *S. minor* was obtained from Chonbuk National University, Korea and *F. solani, F. oxysporium* was obtained from Nepal Agricultural Research Council, Nepal. Potato Dextrose Agar (PDA) supplemented with streptomycin 50ug/ml and Potato Dextrose Broth (PDB) was usedto culture the fungi.

### Morphological and Molecular identification

Isolates were identified as *Trichoderma* based on colony characteristics, growth rate andstructure of mycelium, conidiophores, phialides and conidia as described by Gams and Bissett [9]. Continuous maintenance of isolated *Trichoderma* was achieved by regular transfer on PDA slants under aseptic conditions to keep the culture fresh and viable.

### The invasive growth assay

The dual plate culture experiments were performed as per the techniques developed by Morton and Stroube [10]. Mycelial plugs (5 mm diameter) of actively growing stage were placed opposite to *S. minor, F. oxysoprium* and *F. solani,* respectively. The experiments were done in triplicates. In control petri plates, sterile agar blocks were placed opposite to the pathogen plugs instead of the *Trichoderma* isolates. The plates were incubated at $25\pm2^0$C for 6 days. Measurements of the growth of *Trichoderma* mycelia towards the pathogen, growth of pathogen towards *Trichoderma* and the pathogen alone towards agar plug were done daily. The percent inhibition of the pathogen's radial growth was calculated in relation to growth of the controls as follows:

L = [(C – T)/C] x 100; L - Inhibition of radial mycelial growth of pathogen;

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C - Radial growth measurement of the pathogen in control; T - Radial growth of the pathogen in the presence of *Trichoderma* isolates [11, 12].

**Ability of *Trichoderma*to promote seeds germination**

The *Trichoderma* isolates were cultured on PDB in shaking incubator at 180 rpm for 7 days. The mixture was filtered through muslin cloth to remove the mycelial fragments from the conidial suspension. The conidial concentration was adjusted to $5\times10^6$ conidia/mL after counting conidia in a hemocytometer. 1ml of conidial suspension was pippetedto a 2.0-mL plastic tube. Thirty broad leaf mustard seeds var. Marpha (Himalayan Agro Enterprises Center) were surface-sterilized with 0.1 % sodium hypochlorite and then soaked in the conidial suspension for 60 min. Seedssoaked in sterilized water were used as control. Then, the seeds of eachtreatment were transferred onto the Petri dishes (9 cm diameter) with filter papers soaked in sterile water. 10 seedswere added to each petridish, and incubated at 28°C under dark for five days.Total number of seeds germinated in each dish wascounted. At the end of experiment, length of the radical, hypocotyl, germination percentage and seed vigour index was calculated. Seed vigour index (SVI) for each treatment wascalculated using the formula as described by Baki and Anderson [13]:

$$SVI = (R + H) \times G$$

where, G, H and R represent percentage of germinated seeds, average length of the hypocotyl and average length of the radicle, respectively.

**Pot experiment**

Soil was collected from the A horizon of common farm around Dhulikhel. The soil was air-dried, passed through a 4-mm sieve and mixed with cocopeat and ash in the ratio of 2:1:1 v/v. The soil and cocopeat was autoclaved three times at 121°C for 30 minute prior to use [14].

Broad leaf mustard seedlings were treated with 10ml of each *Trichoderma* isolates of spore suspension of $5x10^6$ conidia/ml.The pots were arranged in a greenhouse (14 ± 2°C) in a randomized block designed in five replicates and watered as needed. Growth parameters leaf number, root length, shoot length, wet weight and dry weight were recorded.

**Statistical analysis**

Data obtainedwere analyzed using MS Excel 2010.

**RESULTS AND DISCUSSION**

Isolates of *Trichoderma* were examined macroscopically and microscopically. They formed colonies with white mycelia, becoming green when forming conidia andconidiophores as shown in Figure 1.

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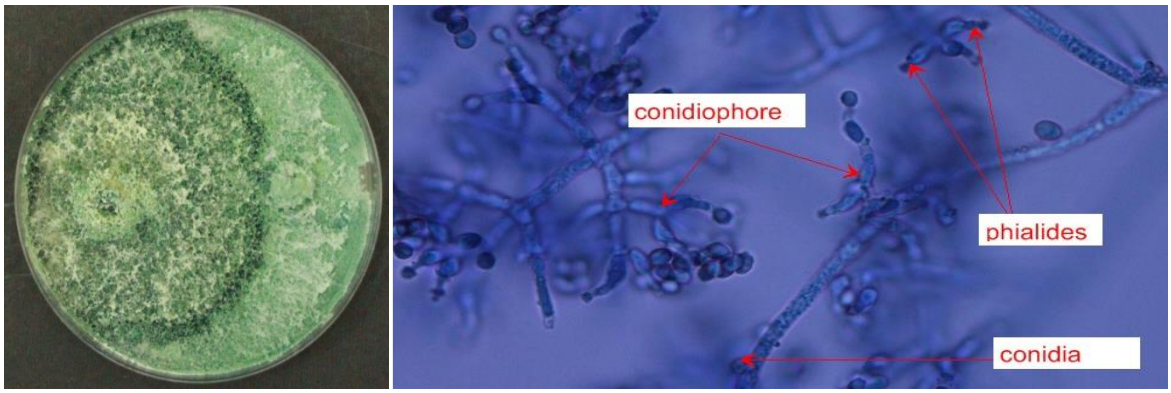


**Figure 1.** Morphology and microscopic analysis of *Trichoderma*

Almost all isolates fully colonized the petriplate after 72 hours The results of dual culture indicated that two *Trichoderma* isolates i.e. BC and KB significantly inhibited the growth of *S. minor* at 100% restricting it almost completely in plates (Figure 2) as compared to the control consisting of *S. minor* growing alone. Isolates DH and TH suppress partial growth of *F. solani* by 64.74% and 70.94% respectively(Figure 3).Isolates HA and Y restrain the growth of *F. oxysoprum* by 85.12% and 85.90% respectively (Figure 4).

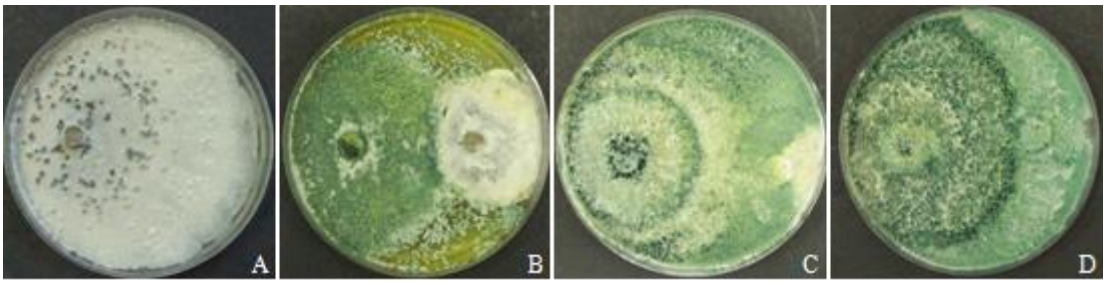


**Figure 2.** Antagonistic effect of isolate BC and KB on *S. minor*, A: *S. minor* control; B: Positive control; C: contact between BC and *S. minor*; D: contact between isolate KB and *S. minor*

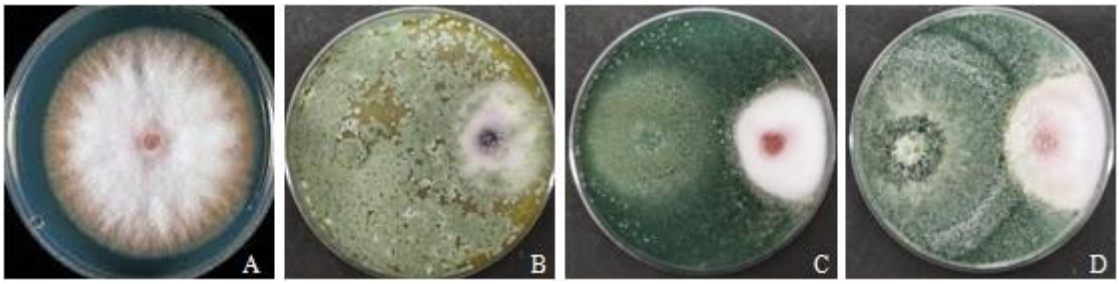


**Figure 3.** Antagonistic effect of isolate HA and Y on *F. oxysporum*, A: *F. oxysporum* control; B: Positive control; C: interaction between HA and *F. oxysporum*; D: interaction between isolate Y and *F. oxysporum*

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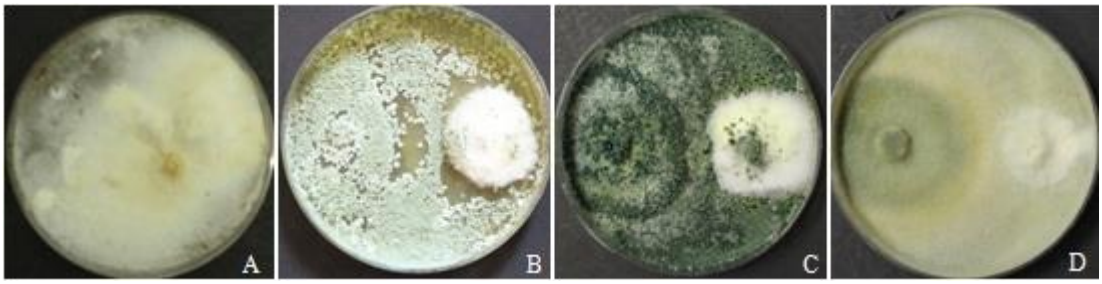


**Figure 4.** Antagonistic effect of isolate DH and TH on *F. solani*, A: *F. solani* control; B: Positive control; C: interaction between DH and *F. solani*; D: interaction between isolate TH and *F. solani*

From in vitro experiment, *Trichoderma* species were capable to inhibit the growth of *S. minor*, *F. oxysoprum* and *F. solani* significantly by growing much faster than the pathogenic fungus, thus competing efficiently for limited nutrients and space. This was in accordance to the findings of Ommati and Zaker (2012) who evaluated the antagonistic capacities of *Trichoderma* species against *F. solani* and *F. oxysporum*[15]. Furthermore, Cotxarrera and Segarra reported *T. asperellum* as an efficient biological control agent in controlling *F. oxysporum.* Starvation has been regarded as the most common cause of death for microorganisms, so that competition for limiting nutrients resulted in biological control of fungal phytopathogens [16]

*Trichoderma spp*. parasitize and inhibit the plant pathogens by mycoparasitism, secretion of lytic, proteolytic enzymes, diffusible or volatile or secondary metabolites and ABC transporter membrane pumps that disrupts the host cell wall and cytoplasm [17][18]. In addition they secrete metabolites; polyketides, pyrones, paracelsin, terpenes, gliotoxin and harzianolide that act against peripheral pathogens [19]. The production of antibiotics includes trichodermin, trichodermol, herzianolide, ethylene and formic aldehyde [20, 21].

The effect of *Trichoderma* isolates on plant growth and development have important economic implications such as shortening the plant growth period aswell as improving plant vigour to overcome biotic and/or abiotic stresses resulting in increased plant productivity and yields. The effect of various *Trichoderma* treatments on different growth parameters are shown in Table 1.

**Table 1.** Effect of different treatments on germination efficiency, Germination Percentage, Seed Vigour Index and plant biomass of (*Brassica juncea var. Marpha*) under laboratory conditions. Mean ± SE (n=30)

| Treatment | Root Length | Shoot length | Plant wet weight | Plant dry wt | %GP | SVI |
|---|---|---|---|---|---|---|
| *T. pleuroticola* | 1.91±0.3 | 1.63±0.13 | 0.098±0.008 | 0.032±0.023 | 100 | 354 |
| Water Control | 1.94±0.33 | 1.44±0.24 | 0.060±0.009 | 0.014±0.003 | 70 | 236.6 |
| BC | 1.39±0.19 | 1.78±0.28 | 0.065±0.016 | 0.009±0.000 | 80 | 253.6 |
| DH | 1.82±0.09 | 1.38±0.06 | 0.080±0.008 | 0.008±0.001 | 70 | 224 |
| EO | 3.15±0.67 | 1.82±0.12 | 0.129±0.008 | 0.011±0.000 | 100 | 497 |
| GS | 2.16±0.78 | 1.22±0.26 | 0.085±0.013 | 0.009±0.004 | 80 | 270.4 |
| GK | 2.67±0.45 | 1.52±0.09 | 0.081±0.003 | 0.007±0.001 | 80 | 335.2 |

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|---|---|---|---|---|---|---|
| HA | 3.02±0.4 | 1.38±0.09 | 0.084±0.007 | 0.009±0.000 | 80 | 352 |
| KB | 2.00±0.23 | 1.50±0.07 | 0.094±0.014 | 0.009±0.001 | 90 | 315 |
| TH | 2.02±0.24 | 1.83±0.13 | 0.110±0.008 | 0.009±0.001 | 70 | 269.5 |
| TV | 2.30±0.29 | 1.54±0.06 | 0.096±0.010 | 0.009±0.001 | 80 | 307.2 |
| Y | 1.97±0.68 | 1.19±0.15 | 0.073±0.017 | 0.009±0.001 | 80 | 252.8 |

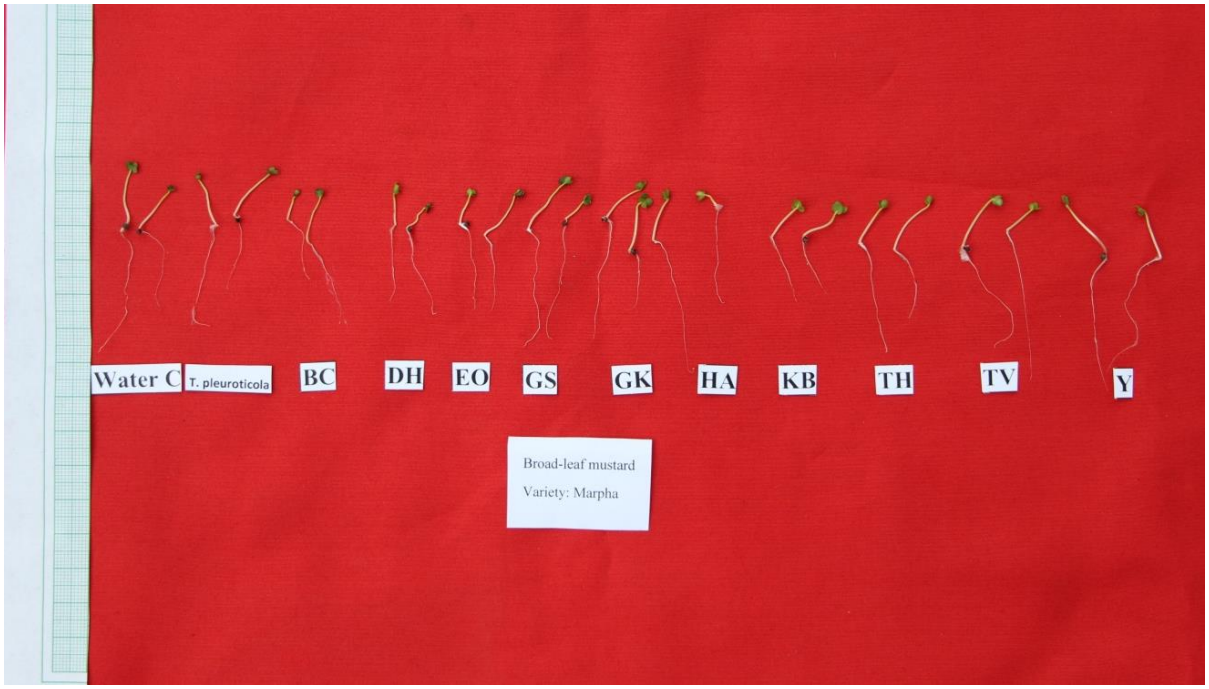


**Figure 5.** Effect of different *Trichoderma* treatments on germination efficiency of *Brassica juncea var. Marpha*

This fungus may produce growth promoting phytohormones like indole acetic acid (IAA) or auxin analogues and vitamins [22][23]. In addition, production of organic acids such as gluconic citric and fumaric acid (scileo) reduces the soil pH resulting in solubilization of phosphates. Micronutrients and minerals such as Fe, Mn, and Mg have important role in plant growth and secretion of diffusible metabolites. There was a significant difference ($P<0.05$) in shoot and root length in greenhouse experiment and plant wet weight in seedling assay.

Some *Trichoderma* strains are capable of enhancing plant biomass production, promoting lateral root growth through an auxin dependent mechanism [18] and/or are able to produce indole-3-acetic acid (IAA) or auxin analogues [19].

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**Table 2.** Effect of *Trichoderma* isolates on different growth parameters in broad leaf mustard in greenhouse pot trials. Mean ± S.D (n=5)

| Treatment | Length of root | Length of shoot | No. of leaves | Root wet wt | Shoot wet wt | Root dry wt | Shoot dry wt |
|---|---|---|---|---|---|---|---|
| Water control | 13.74±0.61 | 15.16±0.82 | 4.40±0.24 | 1.17 | 2.05 | 0.28 | 0.39 |
| *T. pleuroticola* | 15.98±0.43 | 15.62±0.66 | 4.40±0.24 | 0.85 | 2.49 | 0.34 | 0.89 |
| BC | 13.32±2.03 | 14.32±0.92 | 4.80±0.37 | 0.99 | 2.03 | 0.48 | 0.85 |
| DH | 17.36±2.00 | 1.65±0.74 | 4.2±0.20 | 1.46 | 2.25 | 0.38 | 1.32 |
| EO | 14.1±0.76 | 14.86±0.81 | 4.8±0.2 | 0.60 | 2.70 | 0.30 | 1.04 |
| GS | 17.8±1.50 | 15.86±0.81 | 4.6±0.24 | 1.28 | 2.23 | 0.95 | 0.34 |
| GK | 14.38±0.82 | 15.9±0.53 | 4.2±0.20 | 0.89 | 1.81 | 0.61 | 0.29 |
| HA | 17.92±0.72 | 13.54±0.79 | 5.00±0.00 | 1.15 | 1.73 | 0.65 | 1.14 |
| KB | 15.14±0.73 | 12.9±1.53 | 4.6±0.24 | 0.72 | 1.86 | 0.26 | 1.25 |
| TH | 18.14±1.32 | 14.76±1.20 | 4.2±0.37 | 0.41 | 1.98 | 0.22 | 0.33 |
| TV | 15.4±0.80 | 15.48±0.73 | 5±0.55 | 1.01 | 2.14 | 0.65 | 0.30 |
| Y | 15.92±0.77 | 15.4±0.74 | 4±0.45 | 2.68 | 2.35 | 0.28 | 0.29 |


**Figure 6.** Effect of *Trichoderma* isolates on growth parameters of root of broad leaf mustard in greenhouse pot trials

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**Figure 7.** Effect of *Trichoderma* isolates on growth parameters of broad leaf mustard in greenhouse pot trials

## CONCLUSION

The results in this study are highly promising. They support the potential of *Trichoderma* as suppressor of pathogen growth in in-vitro condition and as growth promoter in in-vitro and in-vivo condition. Further field investigations are ongoing for using *Trichoderma* as biocontrol agent to fight against *S. minor* causing lettuce drop.

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