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# Bioformulation of *Trichoderma harzianum* Rifai for management of soybean stem-rot caused by *Rhizoctonia solani* Kuhn

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**ABSTRACT:** ***In vitro*** **osmoticant (manitol) amended and osmoticant free media of potato dextrose broth (PDB), modified Richard's broth (MRB) and Czapek dox broth (CDB) were tested for biomass production of *Trichoderma harzianum* Rifai. Osmoticant amended MRB was found best for production of maximum sporulation, cfu and dry weight of biomass of the antagonist. Osmoticant amended media produced higher biomass of *T. harzianum* than osmoticant free media. Growth and sporulation of *T. harzianum* was assessed in different carrier *viz.* starch, talc and molasses enriched charcoal powder (MECP) based formulation. Starch and MECP based formulation of *T. harzianum* showed increased trend in sporulation of the antagonist upto 60 days, but after this, viability of *T. harzianum* was abruptly decreased. Talc based formulation-exhibited gradual declining trend in multiplication and sporulation of *T. harzianum* after 30 days onwards. Among the nine formulation evaluated for growth and sporulation, starch based formulation alone performed best upto 60 days, followed by MECP based formulation. But after 60 days onwards, talc based formulation @ 3: 1 dose showed highest sporulation and maximum cfu, followed by starch formulation @ 1: 1 dose. The overall performance of talc-based formulation was better for growth and sporulation of the antagonist. Potentiality of *T. harzianum* as seed treatment with talc based *T. harzianum* and chemical were assessed field condition against *Rhizoctonia solani* causing stem rot disease of soybean. Lowest disease index and highest % seed germination was observed when seeds were treated with formulation of *T. harzianum* + talc + osmoticant formulation with enhanced plant vigour and yield of the crop.**

**KEY WORDS:** Biomass, osmoticant, *Rhizoctonia solani*, soybean, stem rot, *Trichoderma harzianum*

## INTRODUCTION

*Rhizoctonia* solani Kuhn, causing soybean stem-rot, is one of the most destructive pathogen causing 40-50 per cent yield loss. Chemical control of the pathogen is not economical besides having environmental hazards. Biological seed treatment has been found to be an efficient method for protection to crop plant against various diseases (Chao *et al.*, 1986) and provide enhanced plant growth (Mukhopadhyay, 1987; Jang *et al.*, 1993). Biological seed treatments are more economical as compared to other field application systems, easy to adapt, and control disease right from sowing to harvesting. The present investigation was carried out to exploit a suitable method for biomass production as well as to test the potentiality of few carrier based material for enhancing the effectiveness of seed treatment with the bioagent under field condition.

## MATERIALS AND METHODS

Three different media *viz.* Potato dextrose broth (PDB), modified Richard's broth (MRB) and Czapek dox broth (CDB) was used to study the growth and sporulation of *Trichoderma harzianum*. Two sets, of media one amended with 6 per cent (w/w) osmoticant (manitol as osmoticant was used to decrease the water potential of media), which was added aseptically after sterilization and the other free from osmoticant were prepared. One hundred ml of each medium was taken into two fifty-ml of conical flasks and sterilized at 121°C for 30 minutes.

**Production of inoculum**

Inoculum of *T. harzianum* was produced by growing the fungus on PDA plate for 7 days. Spores removed by scraping with spatula were suspended in distilled water by mixing with a stir bar, and then pelleted in centrifuge. It was then suspended in distilled water. This suspension was further diluted into 1ml of ($1X\ 10^7$ spore $ml^{-1}$) aliquots, placed in sterile micro centrifuge tube and stored in the refrigerator until use. The cultures of *T. harzianum* were grown in 250ml conical flasks containing 100 ml. of each medium. Each flask with above treatment was inoculated with 1 ml of spore suspension containing $1x10^8$ spores and incubated in a BOD incubator. The experiment was arranged in factorial completely randomized block design (CRD) with five replication of each medium. Fourteen days after incubation spore and mycelium from each of the treatment was combined and homogenized by using mechanical shaker for 30 minutes. Homogenates were centrifuged for 15 minutes 20,000 rpm and supernatant was discarded. The pellet was then allowed to dry in an incubator for 2 days as thin layer in Petri-dishes. The dry weight of biomass and spore concentration of *T. harzianum* was measured with the help of electronic balance and haemocytometer (seven fold dilution), respectively. The number of colony forming unit (cfu) was determined by using dilution plate technique on PDA medium. The best treatment combination found in the present investigation was selected for biomass production of *T. harzianum*.

**Shelf life study**

To study the shelf life of *T. harzianum* in different carriers *viz.* starch (Revive), talc and molasses enriched charcoal powder (MECP) were used and biomass of *T. harzianum* was extracted from the above best medium. Carrier like starch and talc were collected as Revive and talc powder respectively from commercial sources, however, MECP was powdered by mixing 4 g of molasses with 100 g of powdered charcoals. For each carrier, three doses *viz.*, 1:3, 1:1 and 3:1 were obtained by mixing 5, 10 and 15g of dried biomass of *T. harzianum* along with 15, 10 and 5 of carrier material, respectively.

All the carrier based formulation used for testing the growth of *T. harzianum* were packed into sterilized polypropylene bags (8.0 x 6.5$cm^2$) and incubated at 4°C for 120 days and sporulation and cfu were observed at an interval of 30 days, starting from 0 days onwards.

The best dose of the different carrier based formulations was used after 30 days of incubation (DOI) for seed treatment of soybean for management of stem rot disease. From 0 up to 60 DOI and from 90 to 120 DOI the storage period was considered as short storage period and prolong storage period, respectively. The experiment was arranged in factorial completely randomized block design (CRD) each treatment repeated for thrice.

The effectiveness of different formulation of *T. harzianum* as seed treatment against stem rot of soybean, was evaluated during *kharif* season of year 1999-2000 in the experimental plot (size: 2 X 1m) of Department of Plant Pathology, AAU, Jorhat. All the plots, except in the uninoculated control plot were inoculated with 15 days old culture of *R. solani* grown on 4 per cent maize meal sand medium (MSM) @ 2 per cent (w/w). Healthy soybean seeds (cv. PK-564) were treated with *T. harzianum* based formulation which was selected in terms of sporulation and cfu at the prolong days of storage, and with chemicals (Thiram). Seeds were sown 24h after inoculation of *R. solani* with a spacing of 12 x

45cm². The experiment was arranged in randomized block design (RBD), replicating each treatment 4 times.

Plant showing typical symptoms of stem rot during the growth period were rated by using; 1 - 5 scales; 1 - no symptoms; 2 - one or few pin point dark spot; 3 - necrotic lesions with 25 – 50 per cent of the stem girdled; 4 - necrotic lesions with 50 – 75 per cent of the stem girdled; 5 - plants with 100 per cent of the stem girdled. A disease index was calculated by following the method given by Dutta and Das (1999) and Dutta *et al.* (2000).

Observation on per cent seed germination (7 days after sowing), plant height (cm), dry weight of roots and shoots (g) (at the time of final harvest) and grain yield (q/ha) were also recorded.

## RESULTS AND DISCUSSION

MRB + manitol supported highest sporulation (227.9 x $10^6$ $g^{-1}$) and cfu (157.9 x$10^7$ $g^{-1}$) and dry weight of biomass (519.0 mg 100 $ml^{-1}$) of *Trichoderma* followed by CDB + manitol and MRB without manitol (Table1). But manitol free PDB proved to be a poor medium. Among all the media, MRB with or without osmoticant was found to be the best for sporulation (227.9 X $10^7$ spores/g), cfu (157.9 X $10^7$cfu/g) and biomass production (519.0X mg/100ml) of *T. harzianum*, followed by CDB. PDB can be designated as a poor medium in comparision to MRB and CDB. Significantly higher biomass of *T. harzianum* was found in media amended with an osmoticant than the unamended media. Addition of osmoticant markedly decreased the osmotic potential of a medium (Harman *et al.*, 1991). Low osmotic condition might be helpful for the higher sporulation, cfu and biomass production of *T. harzianum* as observed by Crowe and Crowe (1986) in case of Yeast and Euglena.

Significantly higher sporulation (83.33 X $10^6$ spores/g)) and cfu (59.00 X$10^7$cfu/g) were found in formulation of *T. harzianum* + Starch at 3:1 dose than other formulations during short storage period (up to 60 days (Table 2). This was followed by *T. harzianum* + MECP (3:1 dose). *T. harzianum* + talc (1:3 dose) was found to be less efficient as compared to other formulations during short storage period. But during prolong storage period (90 days to 120 days), *T. harzianum* + talc (1:3 dose) showed a significantly higher sporulation (25.67 X$10^6$spores/g) and cfu (12.3 X$10^7$ cfu/g) than rest of the formulations, followed by starch + talc at 1:1 dose. However, these two treatments were statistically on par. The formulation of *T. harzianum* with MECP and starch at 3:1 dose was found to be poor for both sporulation and cfu during the prolong storage

**Table 1. Growth, sporulation and dry weight of biomass of *T. harzianum* in different media**

| Media | X$10^6$ spores/g* | X$10^7$cfu/g * | Dry weight of biomass (mg/100ml)* |
|---|---|---|---|
| Potato Dextrose Broth (PDB) | 13.45 | 1.60 | 221.50 |
| Modified Richards Broth (MRB) | 139.60 | 62.70 | 469.00 |
| Czapek Dox Broth (CDB) | 49.60 | 22.40 | 422.00 |
| PDB + Mannitol | 27.92 | 3.64 | 307.50 |
| MRB + Mannitol | 227.90 | 157.90 | 519.00 |
| CDB+ Mannitol | 146.20 | 96.90 | 475.50 |
| SEM (±) | 3.69 | 3.19 | 5.37 |
| CD (P = 0.05) | 7.24 | 6.26 | 10.53 |

*Values are mean of two years data.

**Table 2. Shelf life of *Trichoderma harzianum* in different carrier based formulation (without osmoticant) in storage**

| Formulation | X10$^6$ spore/g days after incubation | | | | | | | | | X10$^7$ cfu/g |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 | 0 | 30 | 60 | 90 | 120 |
| *T. h*+ Strach (1:3) | 29.67 | 32.33 | 36.67 | 20.67 | 16.33 | 17.30 | 20.90 | 23.67 | 8.20 | 5.80 |
| *T .h* + Strach (1:1) | 49.00 | 54.67 | 59.00 | 22.00 | 17.33 | 28.50 | 32.60 | 37.33 | 9.10 | 6.30 |
| *T. h.*+ Strach (3:1) | 71.33 | 79.67 | 83.33 | 18.67 | 11.67 | 42.60 | 54.70 | 59.00 | 6.20 | 2.40 |
| *T. h.*+ Talc (1:3) | 35.67 | 30.00 | 28.67 | 25.67 | 20.00 | 22.40 | 18.10 | 14.20 | 12.30 | 8.60 |
| *T. h.* + Talc (1:1) | 57.00 | 47.33 | 43.67 | 23.00 | 16.67 | 33.37 | 29.40 | 16.90 | 10.10 | 6.20 |
| *T. h.*+ Talc (3:1) | 86.33 | 70.33 | 57.33 | 20.33 | 12.67 | 56.20 | 45.30 | 20.30 | 8.40 | 3.20 |
| *T. h.*+ MECP(1:3) | 26.00 | 31.00 | 33.33 | 17.33 | 11.33 | 16.10 | 19.60 | 21.10 | 6.00 | 3.60 |
| *T. h.*+ MECP (1:1) | 44.67 | 48.33 | 85.00 | 19.67 | 14.00 | 24.70 | 30.50 | 34.20 | 7.80 | 5.10 |
| *T. h.*+ MECP (3:1) | 65.00 | 72.00 | 75.33 | 14.33 | 9.67 | 40.80 | 51.90 | 54.80 | 5.10 | 1.70 |
| SEM (±) | 3.45 | 2.54 | 3.25 | 1.11 | 1.16 | 2.49 | 2.28 | 2.93 | 0.97 | 0.60 |
| CD (P=0.05) | 7.24 | 5.34 | 6.81 | 2.33 | 2.44 | 5.23 | 4.78 | 6.15 | 2.03 | 1.26 |

*T. h.*: *Trichoderma harzianum*, MECP: Molasses Enriched Charcoal Powder

**Table 3. Shelf life of *T. harzianum* in different carrier based formulation (with osmoticant) in storage**

| Formulation | X10$^6$ spore/g days after incubation | | | | | | | | | X10$^7$ cfu/g |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 | 0 | 30 | 60 | 90 | 120 |
| *T. h.* + Starch (1:3) | 42.00 | 49.33 | 52.33 | 31.67 | 23.67 | 29.33 | 33.67 | 37.33 | 17.67 | 12.00 |
| *T. h.*+ Starch (1:1) | 68.00 | 87.33 | 89.33 | 33.00 | 25.00 | 50.33 | 56.67 | 59.67 | 19.00 | 13.00 |
| *T .h.*+ Starch (3:1) | 109.00 | 126.67 | 131.67 | 28.67 | 16.33 | 78.00 | 88.33 | 90.00 | 14.00 | 5.33 |
| *T.h.* + Talc (1:3) | 52.00 | 44.33 | 42.67 | 37.67 | 29.33 | 36.33 | 32.00 | 27.67 | 23.00 | 16.33 |
| *T .h.*+ Talc (1:1) | 92.67 | 76.33 | 61.33 | 34.67 | 24.67 | 60.33 | 51.67 | 36.67 | 20.67 | 12.67 |
| *T .h.*+ Talc (3:1) | 142.33 | 121.00 | 95.00 | 31.00 | 18.67 | 93.00 | 82.67 | 48.33 | 17.67 | 7.00 |
| *T. h.*+ MECP(1:3) | 37.00 | 44.67 | 47.33 | 27.00 | 18.33 | 26.00 | 32.33 | 34.33 | 13.33 | 8.33 |
| *T.h.*+ MECP(1:1) | 60.67 | 77.67 | 83.33 | 30.67 | 21.67 | 46.67 | 53.67 | 56.67 | 15.67 | 10.67 |
| *T.h.*+ MECP(3:1) | 102.33 | 114.00 | 119.67 | 23.67 | 13.33 | 72.33 | 82.00 | 85.00 | 12.33 | 3.33 |
| SEM (±) | 4.103 | 3.542 | 3.882 | 1.220 | 1.16 | 3.593 | 3.866 | 2.861 | 0.974 | 0 . 6 2 2 |
| CD (P=0.05) | 8.042 | 7.438 | 8.152 | 2.562 | 2.436 | 7.545 | 8.121 | 6.008 | 2.045 | 1.306 |

*T. h.*: *Trichoderma harzianum*, MECP: Molasses Enriched Charcoal Powder

period and both were found statistically on par with each other.

Formulation of *T. harzianum* along with starch and MECP as carrier in all the three doses showed increasing trend of sporulation and cfu at the initial stage / short storage period (Table3). But as the storage time increased both sporulation as well as cfu was abruptly decreased.

In all the formulation with all doses (amended with and without osmoticant) the sporulation and cfu of *T. harzianum* was found high up to short storage period (60 days) and thereafter the sporulation and cfu decreased abruptly except talc with all the doses (Table 3 and Table 4). Though the sporulation and cfu in all the formulations decreases from 60 days of incubation to 120 days, the decreasing trend was less when talc was used as carrier. The overall performance of talc in terms of sporulation and cfu was found better in prolong day of storage with all the three doses, amended with and without osmoticant. Analysis of variance of both sporulation and cfu showed significant 'F' ratios among carriers, doses, days of incubation and also all the interaction (Table 4). Talc as a carrier supported *T. harzianum* to produce maximum sporulation ($43.31 \times 10^6$ g$^{-1}$) and cfu ($26.35 \times 10^7$ g$^{-1}$), this was followed by starch and MECP. This might be due to the fact that starch and MECP formulations contain easily available carbon source, which can be easily taken up by *T. harzianum*, resulting in quick exhaustion of food materials. However, in Talc based formulation food source may be slowly available for the organism and support the population at a safe level for prolonged period. Rama Krishnan *et al.* (1994) and Jayarajan and Nakkeeran (1996) reported that there was a gradual decline in the population of *T. harzianum* in Talc based formulation when stored for 120 days, but still there were sufficient viable propagules.

When doses of carrier for *Trichoderma* were considered individually, 3: 1 dose of all cariers was found superior ($50.27 \times 10^6$ spore g$^{-1}$ and $30.17 \times 10^7$ cfu g$^{-1}$) in comparison to other two doses *viz.* 1: 1 and 1: 3 (Table 4). Sporulaton and cfu, both were found significantly higher ($53.18 \times 10^6$ g$^{-1}$ and $33.67 \times 10^7$ g$^{-1}$, respectively) at 30 days of incubation than the other days of incubation. Minimum sporulation and cfu ($14.41 \times 10^6$ spores ml$^{-1}$ and $4.77 \times 10^7$ cfu g$^{-1}$, respectively) was recorded at 120 days of incubation. Similar trend was also observed in osmoticant amended and osmoticant free media. This might be due to the availability of food materials in the initial stage, *T. harzianum* multiplies profusely, but as the food materials become limited for growth of the organism, its population starts declining.

In the field condition per cent seed germination (76.04) and shoot and root length (cm) and yield (q/ha) were significantly higher with lower disease index (1.8) in the plot where seeds were treated with *T. harzianum* incorporated with talc + manitol (Table 5). This was followed by seed treatment with Thiram (0.3%). Seeds treated with *T. harzianum* along with osmoticant amended talc showed significant increase in seed germination (76.04%) and shoot and root length and decrease in disease index (1.8) over seeds treated with *T. harzianum* + talc alone. Spores of *T. harzianum* from osmoticant amended medium contains higher level of trehalose, which help the organism to tolerate low osmotic potential and survive longer in water stress condition (Harman *et al.*, 1991). In the present study spores obtained from *T. harzianum* in Talc + manitol might have higher population with vigorous growth and release more antibiotic like substances against the pathogen and more growth regulator (Chang *et al.*, 1986) in favour of plants resulting in minimum disease index and increased growth and yield. Lowest seed germination (31.25%), plant height (58.20 cm), dry weight of roots and shoots (0.17 and 3.39g plant$^{-1}$) and grain yield (8.14 q ha$^{-1}$) was recorded when *R. solani* was inoculated alone in soil (Table 5).

The present investigation shows encouraging results by using bioformulation of *T. harzianum* with different carriers + osmoticant as seed treatment against soybean stem rot caused by *R. solani*

**Table 4. Mean spore concentration and colony forming unit of *T. harzianum* on different doses of carriers at different days of incubation**

| | Mean spores ($X10^6$ spores/g) | | Mean spores ($X10^6$ spores/g) | |
|---|---|---|---|---|
| | Without osmoticant | Amended with osmoticant | Without osmoticant | Amended with osmoticant |
| Carriers: | | | | |
| Starch | 40.15 | 61.62 | 23.64 | 39.29 |
| Talc | 43.31 | 64.24 | 26.35 | 41.93 |
| Molasses Enriched Charcoal Powder | 35.53 | 54.62 | 21.53 | 36.78 |
| SEM (±) | 1.179 | 1.288 | 0.913 | 1.023 |
| CD (P=0.05) | 2.313 | 2.524 | 1.789 | 12.005 |
| Doses: | | | | |
| 1:3 | 26.31 | 38.89 | 14.20 | 25.24 |
| 1:1 | 38.42 | 59.09 | 20.83 | 37.80 |
| 3:1 | 50.27 | 79.51 | 30.17 | 51.95 |
| SEM (±) | 1.179 | 1.288 | 0.913 | 1.023 |
| CD (P=0.05) | 2.313 | 2.524 | 1.789 | 2.005 |
| Days of Incubation: | | | | |
| 0 | 51.41 | 79.70 | 31.37 | 54.70 |
| 30 | 53.18 | 83.70 | 33.67 | 57.72 |
| 60 | 50.08 | 79.29 | 31.28 | 52.85 |
| 90 | 20.18 | 30.89 | 8.13 | 17.04 |
| 120 | 14.41 | 21.22 | 4.77 | 9.85 |
| SEM (±) | 1.523 | 1.663 | 1.178 | 1.321 |
| CD (P=0.05) | 2.985 | 3.259 | 2.309 | 2.588 |
| SEM (±) between interaction of carriers and doses | 2.044 | 2.231 | 1.581 | 1.772 |
| CD (P=0.05) | 4.005 | 4.372 | 3.098 | 3.473 |
| SEM (±) between interaction of carriers and days of incubation | 2.638 | 2.879 | 2.041 | 2.287 |
| CD (P=0.05) | 5.171 | 5.644 | 3.999 | 4.483 |
| SEM (±) between interaction of doses and days of incubation | 2.638 | 2.879 | 2.041 | 2.287 |
| CD (P=0.05) | 5.171 | 5.644 | 3.999 | 4.483 |
| SEM (±) between interaction of carriers, doses and days of incubation | 4.569 | 4.988 | 3.534 | 3.962 |
| CD (P=0.05) | 8.957 | 9.776 | 6.928 | 7.766 |

**Table 5. Effect of seed treatment with *T. harzianum* along with different carriers on seed germination, disease index, plant growth parameter and yield of soybean**

| Treatment | Seed Germn. (%) | Disease index | Plant ht. (cm) | Root dry wt/plant (g) | Shoot dry wt/plant (g) | Grain yield (q/ha) |
|---|---|---|---|---|---|---|
| $T_1$ Untreated seeds + no *R. solani* | 79.17 (62.84) | 1.2 | 62.25 | 0.91 | 10.24 | 24.58 |
| $T_2$ Untreated seeds + *R. solani* | 31.25 (33.99) | 4.7 | 58.20 | 0.17 | 3.39 | 8.14 |
| $T_3$ *T. h.* (biomass) treated seeds + Talc + Manitol + *R. solani* | 76.04 (60.70) | 1.8 | 61.50 | 0.84 | 10.03 | 24.07 |
| $T_4$ *T. h.* (biomass) treated seeds + Talc + *R. solani* | 63.54 (52.86) | 2.9 | 60.65 | 0.59 | 9.12 | 21.89 |
| $T_5$ *T. h.* (in broth) treated seeds + Talc + *R. solani* | 55.21 (47.98) | 3.6 | 60.45 | 0.42 | 8.44 | 20.26 |
| $T_6$ Seed treated with Thiram (0.3%) *R. solani* | 73.96 (59.32) | 1.9 | 61.0 | 0.81 | 9.85[a] | 23.64 |
| SEM ± | 2.01 | 0.15 | NS | 0.05 | 0.10 | 0.69 |
| CD (P = 0.05) | 4.29 | 0.32 | | 0.11 | 0.22 | 1.46 |

Figures in parentheses are angular transformed values.

Figures followed by same letters are not significant at P=0.05.

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