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Standardization of optimal moisture level on population density, longevity and multiplication of drought tolerant *T. harzianum* isolates on de-oiled cake of Neem

Prashant Singh, Ramji Singh, Madhu GS and Vinit Pratap Singh

Abstract
Trichoderma species naturally occurring antagonistic microorganisms has performed great potential to antagonize plant pathogens. Hence, biocontrol involving the use of such beneficial microorganisms for plant protection is being considered as a viable substitute to reduce the use of chemical pesticides. These microorganisms need to be multiplied and kept viable often for long period under storage. This study aim to study multiplication and longitivity of *Trichoderma* isolates on de-oiled neem cake at different moisture regimes. Fifteen isolates of *Trichoderma* were isolated in different region of Utter Pradesh state of India and one isolate IRRI 1 was collected from Internation Rice Research Institute. Neem cake was able to support the highest population dynamics of *T. harzianum* strains at 70 per cent moisture level up to 21 days. Increasing the moisture beyond 70 per cent resulted in declining the population dynamics of *T. harzianum*. These findings suggested that 70 per cent additional moisture in the de-oiled cake of Neem is most appropriate for increasing the population dynamics of *T. harzianum* strains. *T. harzianum* strains *i.e.* IRRI-1, TH-28, TH-26, TH-21, TH-30, TH-3, TH-29 and TH-7 were found to be best performer with regard to their rate of multiplication. Even without additional moisture, the two strains *i.e.* IRRI-1 and TH-28, could sustain their viability at the cake.

Keywords: *Trichoderma harzianum*, cake, neem, strains

Introduction
Trichoderma species are effective antagonists with biocontrol abilities against economically significant plant parasitic soil-borne infections and are found in nearly all soil types (Shahid *et al*., 2014) [6]. It's a free-living fungus found in soil and root ecosystems, and it's extremely interacting in root, soil, and foliar environments. It inhibits pathogen growth, survival, and infections through a variety of methods including antibiosis, mycoparasitism, hyphal contacts, enzyme release, induced host cell resistance, and nutrition and space competition. (Yedidia *et al*., 2003) [7]. Trichoderma spp. are not only effective pathogen antagonists, but they also promote plant growth and root development by colonizing roots as endophyte symbionts, solubilizing minerals and increasing the availability of insoluble nutrients, secreting siderophores, plant growth regulatory materials (phytohormons), vitamins, and enzymes, and so on (Harman *et al*., 2006) [8]. Trichoderma penetrates the epidermis to colonise root surfaces in a robust and long-lasting manner, increasing plant drought tolerance. Drought avoidance through morphological adaptations, drought tolerance through physiological and biochemical adaptations, and accelerated drought recovery are some of the mechanisms involved in Trichoderma's modulation of drought response (Malinowski *et al*., 2000) [5].
Several microorganisms have been floated in the market as biocontrol agents (formulations) over the last few decades, and many low-cost materials have been used as carriers, but the problem is that they do not perform as well in the field as they do in lab and glasshouse experiments, and appear to be less effective. They also have limitations in terms of viability and population reduction during storage and transportation. As a result, there is a demand for low-cost materials that are nutrient-rich, biodegradable, and compatible with other ingredients along with biocontrol agents support. These materials can be used as substrates for mass multiplication and help to sustain the viability and survival of BCAs for longer periods of time during storage and transit. They can also supply nutrition if applied to soil (field & pots) conditions.

Many agro-industrial bioproducts, such as oil cakes of tree-born oilseeds (TBOs) such as Neem, Jatropha, and Mahua, have either gone to waste or been used as less profitable and usable products for centuries. The oil extracted from Neem, Jatropha, and Mahua trees is either used directly as a biofuel or as a raw material for industrial inputs in industries such as cosmetics, agrochemicals, and pharmaceuticals. These trees' oil cakes are either unexploited or underutilised. These oil cakes contain a variety of chemicals, micronutrients, aldehydes, and ketons that may serve as a good source of nutrition for beneficial microorganisms (growth promoters and biocontrol agents) in crop cultivation and thus may be used to mass multiply fungal biocontrol agents (Trichoderma spp.). As a result, value-added products such as biopesticides and plant growth promoters will be developed. For its multiplication, various organic media such as neem cake, coir pith, farmyard manure, and decomposed coffee pulp have been suggested (Saju *et al*., 2002) [2]. Yet reports on the optimum moisture levels of these substrates for high inoculum production of *Trichoderma* spp. are inadequate. Therefore, a study was conducted to evaluate neem cake as substrates and to standardize the optimum moisture levels for mass multiplication and long-term survival of *Trichoderma* spp.

Materials and Methods

Trichoderma Isolation

Extensive collection of soil samples were performed from different rain fed farming situations from the plains of Uttar Pradesh. The approachable locations of plains were selected and visited for soil sampling. Generally healthy plants were selected from standing crop of a location and rhizospheric soil was collected. Plants were gently and carefully uprooted and soil tightly adhering the root was collected, five such samples collected randomly from the crop field were mixed thoroughly and 1/4th of this mixture was used as composite rhizospheric soil sample of the region. For isolation of *Trichoderma harzianum*, fifteen soil samples were collected from fields of fifteen districts of Uttar Pradesh (India).

Collection of oil cakes and designing of experiment: Agro-industrial byproducts *i.e.* oil cake of Neem, which were used during the course of investigation. These materials were collected from local market. The cakes were crushed in a heavy pestle & mortar to prepare a coarse powder (particles of approximately 1.0 mm diameter). The sample's fresh weight (FW) was recorded followed by dry weight (DW) after samples were dried at 80°C for 72 h in oven. The cake moisture was calculated using the following formula.

$$\text{Cake moisture (\%)} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100$$

Desired moisture levels were maintained by adding additional volume of sterilized distilled water to the cakes, while maintaining the moisture, natural moisture available in the cakes were taken into consideration. These aforementioned oilcakes were used after adding additional water to maintain deferent moisture regimes (10% Moisture regime, 20% Moisture regime**,** 40% Moisture regime, 70% Moisture regime, 90% Moisture regime, Control (0% Moisture regime) where no additional water was added). Further this oil cakes were autoclaved at 121.6^{0}C (1.1 kg/cm^2) for 20 minutes. The flasks were allowed to cool at room temperature prior to inoculation. Flasks containing substrates were inoculated with 3-4 days old actively growing culture of *Trichoderma* spp. (2-3 bits of about 5mm size) under aseptic conditions in laminar flow.

The flasks were shaken thoroughly once a day, and incubated at 28±2^{0}C for 30 days. For each treatment, three replicates of flasks were maintained and arranged in a completely randomized manner. Population dynamics of different *Trichoderma* isolates (at 7 DAI, 14 DAI and 21 DAI under different moisture regimes) were determined by serial dilution plate technique.

Determination of population dynamics: The population dynamics of *T. harzianum* was determined by following serial dilution plate technique at the end of incubation period *i.e.* 7, 14, and 21 days after inoculation. For monitoring the growth, 1gm substrate of each inoculated cake was taken from each flask and it was used for CFUs count using PDA through dilution plate technique. One gram of sample (substrate where *Trichoderma* was being grown) was suspended in 10 ml distilled water to make microbial suspension diluted up to 10^{-6}. Finally 1ml of microbial suspension from last serial dilution *i.e.* 10^{-6} was added to sterile Petri dishes (triplicate in completely randomized manner) containing 30 ml of sterilized PDA medium, which was added with pinch of Streptomycin (to check bacterial contamination) when warm and molten. The Petri dishes were then incubated for 5 days at 28±2^{0}C. The density of spores/conidia of *T. harzianum* (CFUs) was measured in laboratory by plate dilution technique (Johnson and Curl, 1972) [1].

Results and Discussion

Screening of *T. harzianum* strains *viz.* IRRI-1, TH-1, TH-3, TH-4, TH-7, TH-11, TH-14, TH-17, TH- 20, TH-21, TH-23, TH-25, TH-26, TH-28, TH-29 and TH-30 were perfermod on Neem cake for their suitability for multiplication at six different moisture regime *i.e.* 0 per cent, 10 per cent, 20 per cent, 40 per cent, 70 per cent and 90 per cent.

Population dynamics of *Trichoderma harzianum* strains at seven day after incubation at different level of moisture in de-oiled cakes of neem: It is clearly indicate in Table No. 1and Fig. 1 that in the Neem cake without addition of any amount of moisture, only two *T. harzianum* strains *i.e.* IRRI-1 and TH-28 could sustain with 0.22×10^7 level of CFUs otherwise rest of other *T. harzianum* strains could not sustain their viability as no CFUs could be retrieved from these strains at 0% moisture. It was also noticed that there was an increasing trend in the populations of *T. harzianum* with increases level of moisture but only upto 70 per cent. At 70 per cent level of moisture *T. harzianum* strain IRRI-1 showed highest level of CFUs (33.50×10^7). The strain TH-28 resulted in 30.00×10^7 followed by TH-26 (28×10^7) level of CFUs but both were significantly lower than IRRI-1. The other strains like TH-21 (25.80×10^7), TH-30 (22.34×10^7), TH-3 (21×10^7), TH-29 (20.60×10^7), TH-7 (18.00×10^7), TH-20 (17.20×10^7), TH-25 (14.20×10^7), TH-23 (12.00×10^7), TH-14 (11×10^7), TH-1 (10.50×10^7) and TH-4 (9.00×10^7) could also exhibited quite good level of population but were significantly lower than TH-28 and TH-26. When all the strains of *T. harzianum* were compared among them self based on the different moisture regimes *i.e.* 0 per cent, 10 per cent, 20 per cent, 40 per cent, 70 per cent and 90 per cent at 7 days of incubation, it was noticed that highest CFUs were noticed at 70 per cent substrate moisture that was significantly higher than the population values exhibited at other moisture regimes. *T. harzianum* strains *i.e.* IRRI-1, TH-28 and TH-26 were noticed as in higher performer with regard to their CFUs level at all the moisture regimes and were significantly higher than rest of the strains.Significant reductions in CFUs was recorded at 90 per cent moisture level in all of these strains of *T. harzianum*.

Population dynamics of *Trichoderma harzianum* strains at fourteen day after incubation at different level of moisture in de-oiled cakes of neem

Data presented in the Table No. 2 and Fig. 2 clearly indicates that in the Neem cake without addition of any amount of moisture, only two strains of *T. harzianum i.e*. IRRI-1 (0.33×10^7) and TH-28 (0.28×10^7) could sustain otherwise rest of other *T. harzianum* strains, TH-1, TH-3, TH-4, TH-7, TH-11, TH-14, TH-17, TH-20, TH-21,TH-23, TH-25, TH-26, TH-29 and TH-30 could not sustain their viability as no CFUs of these strains could be retrieved from the substrate without additional moisture. It was also noticed that there was an increasing trend in the populations of *T. harzianum* with increases level of moisture but only upto 70 per cent. At 70 per cent substrate moisture, *T. harzianum* strain IRRI-1 (61.17×10^7) followed by TH-28 (58.00×10^7) showed highest level of CFUs, both strains were significantly different from each other. Other strains of *T. harzianum i.e.* TH-26 (56.50×10^7), TH-21 (53.33×10^7), TH-30 (51.33×10^7), TH-3 (49.83×10^7), TH-29 (48.33×10^7), TH-7 (45.55×10^7), TH-20 (43.27×10^7), TH-25 (41.57×10^7), and TH-23 (38.67×10^7) also resulted in a considerable high level of CFUs but they were significantly lower than IRRI-1 and TH-28. When all the strains of *T. harzianum* were compared based on the different moisture regimes *i.e.* 0 per cent, 10 per cent, 20 per cent, 40 per cent, 70 per cent and 90 per cent, it was noticed that highest CFUs were noticed at 70 per cent that was significantly higher than the values at other moisture regimes. *T. harzianum* strains *i.e.* IRRI-1, TH-28 and TH-26 were found to be higher performer with respect to their CFUs level at all of these moisture regimes and were significantly higher than rest other strains. Significant reductions in CFUs were noticed at 90 per cent moisture in all of these strains of *T. harzianum*.

Population dynamics of *Trichoderma harzianum* strains at twenty one days after incubation at different level of moisture in de-oiled cakes of neem: Data presented in the Table No. 3 and Fig. 3 clearly indicates that in the Neem cake without addition of any amount of moisture only two *T. harzianum* strains *i.e*. IRRI-1 and TH-28 could sustain with 0.50×10^7 and 0.43×10^7 level of CFUs otherwise rest other *T. harzianum* strains *viz.* TH-1, TH-3, TH-4, TH-7, TH-11, TH-14, TH-17, TH-20, TH-21, TH-23, TH-25, TH-26, TH-29 and TH-30 could not sustain their viability as no CFUs could be retrieved from the cakes without additional amount of moisture. It was also noticed that there was an increasing trend in the populations of *T. harzianum* with increases level of moisture but only upto 70 per cent. At 70 per cent moisture, the maximum level of CFUs was recorded in IRRI-1 strain of *T. harzianum.* (85.03×10^7) and TH-28 (82.83×10^7) which were significantly different from each other. Other strains of *T. harzianum* like, TH-26 (78.80×10^7), TH-21 (75.17×10^7), TH-30 (73×10^7) TH-3 (70.37×10^7) and TH-29 (67.40×10^7) also resulted in a quite high level of CFUs but were significantly lower than IRRI-1 & TH-28 and significantly different among themselves. When all the strains of *T. harzianum* were compared based on the different moisture regimes *i.e.* 0 per cent, 10 per cent, 20 per cent, 40 per cent, 70 per cent and 90 per cent, it was noticed that highest CFUs of all the *Trichoderma* strains were noticed at 70 per cent substrate moisture that was significantly higher than the values at other moisture regimes. *T. harzianum* strains *i.e.* IRRI-1, TH-28 and TH-26 resulted in higher performer with regard to their CFUs level at all of these moisture regimes and were significantly higher than other rest strains. Significant reductions in CFUs were recorded at 90 per cent moisture level in all of these strains of *T. harzianum*. Results indicate that of the sixteen *T. harzianum* strains, tested at different moisture regime, two *T. harzianum* strains *i.e.* IRRI-1 and TH-28 were found to be best performer *i.e.* stress tolerant as even without addition of extra moisture, these two strains could sustain their viability at either Neem cakes. It was also noticed that there was an increasing trend in the populations of *T. harzianum* with increases level of moisture but only upto 70 per cent. With increasing the moisture beyond 70 per cent, the population started declining. The population *T. harzianum* is start declining at 70 percent moisture regimes after 21 days. These findings suggested that 70 per cent additional moisture in the de-oiled cakes of Neem upto 21 dyas is more suitable for highest level of population dynamics of *T. harzianum* strains. In addition to best performing two strains of *T. harzianum i.e.* IRRI-1 and TH-28 some other strains *viz.* TH-26, TH-21, TH-30, TH-3, TH-29 and TH-7 were also found to exhibit quite considerable level of population dynamics at all the moisture regime with similar trends except at 0 per cent moisture where no population of these strains could be noticed. Our findings also supported by (Tomer., *et al*. 2014) [4] used De-oiled cakes of Neem, Jatropha, Mahua and Karanja were tested for their suitability for mass multiplication and longevity of *T. harzianum.* Neem cake maintained with 25% moisture was supported the highest population dynamics and longevity of *T. harzianum in vitro.* A combination of deoiled cake of neem, jatropha, mahua and karanja along with sorghum grains and wheat bran with moisture level 30% were supported highest population dynamics of *T. harzianum* (86.17×10^6 cfu at 30 days period) and shelf life for 150 days (Singh *et al*., 2015) [4]. Neem cake carrier prolongs shelf life of *T. viridae* for 200 days during storage at 28°C with 35.78×106 cfu /g (Zope *et al*., 2019) [9]. Different organic media like neem cake, coir pith, farmyard manure, and decomposed coffee pulp also have been suggested for its multiplication (Saju *et al*., 2002) [2]. With these our findings suggested that 70 per cent additional moisture in the de-oiled cakes of Neem is most appropriate for increasing the population dynamics of *T. harzianum* strains.

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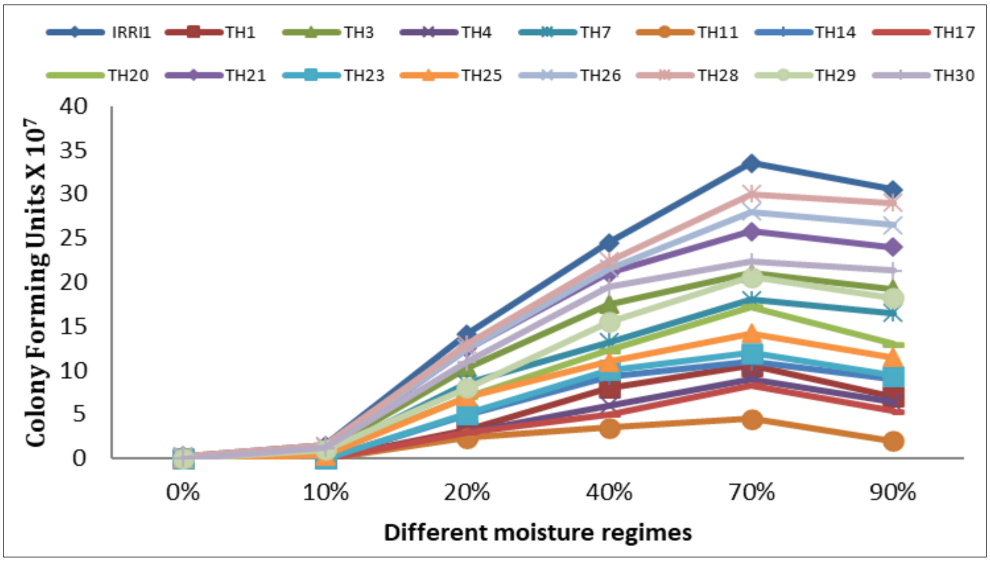

Fig 1: Population dynamics of *Trichoderma harzianum* strains at different level of moisture in de-oiled cakes of neem at seven days of incubation.

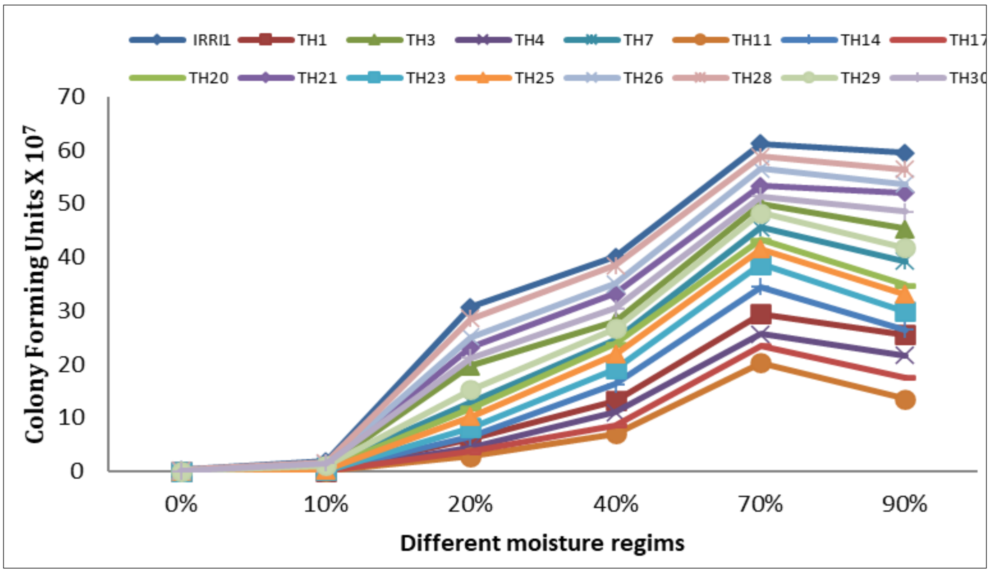

Fig 2: Population dynamics of *Trichoderma harzianum* strains at different level of moisture in de-oiled cakes of neem at fourteen days of incubation.

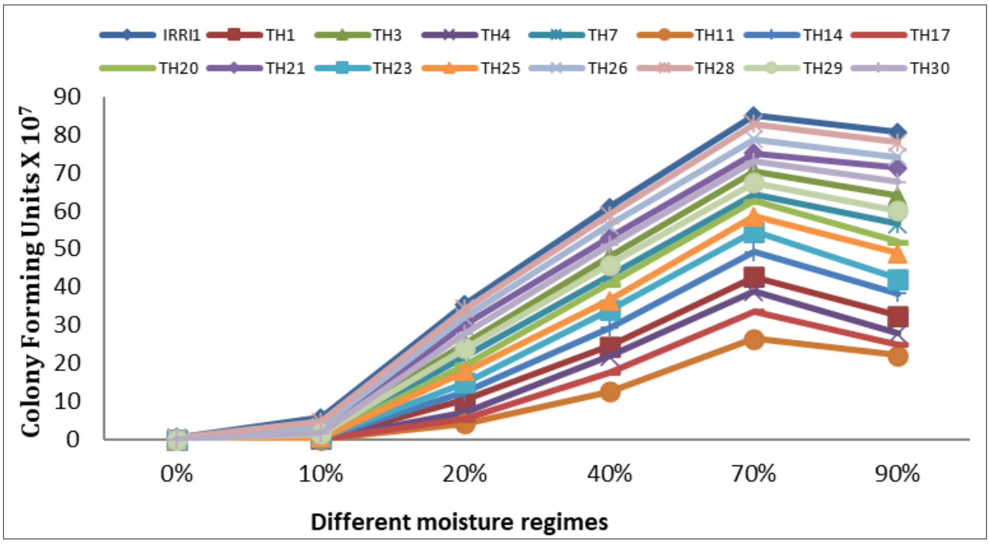

Fig 3: Population dynamics of *Trichoderma harzianum* strains at different level of moisture in de-oiled cakes of neem at twenty one days of incubation.

Table 1: Population dynamics of *Trichoderma harzianum* strains at different level of moisture in de-oiled cakes of neem at seven days of incubation.

Treatments	Strains of *T. harzianum*	Colony forming units of *T. harzianum* at different moisture regime (CFU x 10^7)					
		0%	10%	20%	40%	70%	90%
T_1	*T. harzianum* (IRRI-1)	0.22	1.50	14.10	24.50	33.50	30.50
T_2	*T. harzianum* (TH-1)	0.00	0.00	3.20	8.00	10.50	7.00
T_3	*T. harzianum* (TH-3)	0.00	1.00	10.30	17.50	21.00	19.20
T_4	*T. harzianum* (TH-4)	0.00	0.00	3.00	6.00	9.00	6.40
T_5	*T. harzianum* (TH-7)	0.00	0.50	8.50	13.20	18.00	16.50
T_6	*T. harzianum* (TH-11)	0.00	0.00	2.33	3.50	4.50	2.00
T_7	*T. harzianum* (TH-14)	0.00	0.00	4.90	9.30	11.00	9.00
T_8	*T. harzianum* (TH-17)	0.00	0.00	3.00	5.00	8.33	5.33
T_9	*T. harzianum* (TH-20)	0.00	0.50	7.00	12.23	17.20	13.00
T_{10}	*T. harzianum* (TH-21)	0.00	1.20	12.50	21.00	25.80	24.00
T_{11}	*T. harzianum* (TH-23)	0.00	0.00	5.10	10.00	12.00	9.40
T_{12}	*T. harzianum* (TH-25)	0.00	0.30	7.00	11.00	14.20	11.50
T_{13}	*T. harzianum* (TH-26)	0.00	1.30	12.50	21.50	28.00	26.50
T_{14}	*T. harzianum* (TH-28)	0.22	1.50	13.00	22.40	30.00	29.00
T_{15}	*T. harzianum* (TH-29)	0.00	1.00	8.00	15.50	20.60	18.20
T_{16}	*T. harzianum* (TH-30)	0.00	1.20	11.00	19.45	22.34	21.30
Factors		Factor A (Moisture %)		Factor B(Strains of *T. harzianum*)			Factors A X B
CD@5% level		0.30		0.50			1.23
SE (m)		0.11		0.18			0.44

Table 2: Population dynamics of *Trichoderma harzianum* strains at different level of moisture in de-oiled cakes of neem at fourteen days of incubation.

Treatments	Strains of *T. harzianum*	Colony forming units of *T. harzianum* at different moisture regime (CFU x 10^7)					
		0%	10%	20%	40%	70%	90%
T_1	*T. harzianum* (IRRI-1)	0.50	5.67	35.50	60.87	85.03	80.67
T_2	*T. harzianum* (TH-1)	0.00	0.17	10.33	24.33	42.67	32.33
T_3	*T. harzianum* (TH-3)	0.00	1.67	25.17	48.00	70.37	63.83
T_4	*T. harzianum* (TH-4)	0.00	0.00	7.17	21.70	38.83	27.67
T_5	*T. harzianum* (TH-7)	0.00	0.96	21.80	42.83	64.17	56.53
T_6	*T. harzianum* (TH-11)	0.00	0.00	4.17	12.50	26.33	22.00
T_7	*T. harzianum* (TH-14)	0.00	0.23	12.33	29.17	49.17	38.33
T_8	*T. harzianum* (TH-17)	0.00	0.00	5.37	17.50	33.50	24.85
T_9	*T. harzianum* (TH-20)	0.00	0.74	19.50	40.83	62.53	51.87
T_{10}	*T. harzianum* (TH-21)	0.00	2.33	30.17	52.85	75.17	71.33
T_{11}	*T. harzianum* (TH-23)	0.00	0.40	14.83	33.87	54.53	42.17
T_{12}	*T. harzianum* (TH-25)	0.00	0.48	17.77	36.50	58.53	48.90
T_{13}	*T. harzianum* (TH-26)	0.00	3.33	32.00	56.19	78.80	74.03
T_{14}	*T. harzianum* (TH-28)	0.43	4.57	34.17	58.78	82.83	78.03
T_{15}	*T. harzianum* (TH-29)	0.00	1.37	23.83	45.83	67.40	60.00
T_{16}	*T. harzianum* (TH-30)	0.00	1.67	27.67	51.00	73.00	67.50
Factors		Factor A (Moisture %)		Factor B (Strains of T. harzianum)			Factors A X B
CD@5% level		0.31		0.51			1.26
SE (m)		0.11		0.18			0.45

Table 3: Population dynamics of *Trichoderma harzianum* strains at different level of moisture in de-oiled cakes of neem at twenty one days of incubation.

Treatments	Strains of *T. harzianum*	Colony forming units of *T. harzianum* at different moisture regime (CFU x 10^7)					
		0%	10%	20%	40%	70%	90%
T_1	*T. harzianum* (IRRI-1)	0.50	5.67	35.50	60.87	85.03	80.67
T_2	*T. harzianum* (TH-1)	0.00	0.17	10.33	24.33	42.67	32.33
T_3	*T. harzianum* (TH-3)	0.00	1.67	25.17	48.00	70.37	63.83
T_4	*T. harzianum* (TH-4)	0.00	0.00	7.17	21.70	38.83	27.67
T_5	*T. harzianum* (TH-7)	0.00	0.96	21.80	42.83	64.17	56.53
T_6	*T. harzianum* (TH-11)	0.00	0.00	4.17	12.50	26.33	22.00
T_7	*T. harzianum* (TH-14)	0.00	0.23	12.33	29.17	49.17	38.33
T_8	*T. harzianum* (TH-17)	0.00	0.00	5.37	17.50	33.50	24.85
T_9	*T. harzianum* (TH-20)	0.00	0.74	19.50	40.83	62.53	51.87
T_{10}	*T. harzianum* (TH-21)	0.00	2.33	30.17	52.85	75.17	71.33
T_{11}	*T. harzianum* (TH-23)	0.00	0.40	14.83	33.87	54.53	42.17
T_{12}	*T. harzianum* (TH-25)	0.00	0.48	17.77	36.50	58.53	48.90
T_{13}	*T. harzianum* (TH-26)	0.00	3.33	32.00	56.19	78.80	74.03

T_{14}	*T. harzianum* (TH-28)	0.43	4.57	34.17	58.78	82.83	78.03
T_{15}	*T. harzianum* (TH-29)	0.00	1.37	23.83	45.83	67.40	60.00
T_{16}	*T. harzianum* (TH-30)	0.00	1.67	27.67	51.00	73.00	67.50
Factors		Factor A (Moisture %)		Factor B (Strains of T. harzianum)			Factors A X B
CD@5% level		0.31		0.51			1.26
SE (m)		0.11		0.18			0.45

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