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De-oiled Cakes of Neem, Jatropha, Mahua and Karanja: A New Substrate for Mass Multiplication of *T. harzianum*

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Abstract

De-oiled cakes of four trees born oilseeds (TBOs) viz., Neem, Jatropha, Mahua and Karanja were tested for their suitability for mass multiplication of *T. harzianum* and also that for how long they are able to support the survival of *T. harzianum* with the considerable level of population dynamics of *T. harzianum*. In addition to these four de-oiled cakes, two composts i.e., FYM and Vermicompost were also tested for their suitability and to have a comparison with these de-oiled cakes in supporting population dynamics and longevity of *T. harzianum*. Among four de-oiled cakes *viz* neem, jatropha, mahua and karanja, neem cake was found to be best substrate for supporting the population dynamics and longevity of *T. harzianum in vitro*. Neem cake maintained with 25% moisture was able to support the longevity of *T. harzianum* for more than 105 days with a considerable level of population, whereas Jatropha, Mahua and Karanja cakes could support the longevity of *T. harzianum* up to 90 days only. Mixing of pre grown *Trichoderma harzianum* on potato dextrose broth (PDB) to the four de-oiled cakes resulted in comparatively better population dynamics than that when it was grown on Potato Dextrose Agar.

Keywords: Longevity; Survival; De-oiled cakes; Neem; Jatropha; Mahua; Karanja; Farm yard manure; Vermicompost CFUs; *Trichoderma harzianum*

Introduction

Modern agriculture depends largely on the use of chemical inputs, such as pesticides and fertilizers, to control plant pathogens and to enhance crop yield. No doubt these chemicals enhances agriculture productivity but health concerns and environmental hazards associated with the use of chemical pesticides have resulted in an increasing interest in biological control as a promising alternative or a supplemental way of reducing the use of agro-chemicals. Some naturally occurring soil bacteria and fungi have demonstrated great potential to antagonize crop pathogens, hence, biological control involving the use of such beneficial microorganisms for plant protection is being considered as a viable substitute to reduce the use of chemical pesticides [1]. These beneficial microorganisms need some suitable carrier for their delivery, which can support their life during storage and transportation. Many agro-industrial bio-products such as de-oiled cakes of tree born oils seeds (TBOs) like Neem (*Azadirachta indica*), Jatropha (*Jatropha curcas*), Mahua (*Madhuca longifolia*) and Karanja (*Pongamia pinnata*) which are going waste or used as a less profitable and usable products since centuries. These de-oiled cakes contain several chemicals, micronutrients, and many more constituents which might served as good source of nutrition for beneficial micro-organisms (growth promoter and biocontrol agents) in crop cultivation hence might be exploited for mass multiplication of fungal biocontrol agents (*Trichoderma* spp.). It is worthwhile to mention that these cakes themselves too possess biopesticidal properties [2]. Available mass culture (formulations) in the market generally shows poor efficacy after application in the crop field. This is probably due to long duration taken in transportation from manufacturing unit to farmers. The mass cultures made at industrial scale are generally talc based; talc has no nutritional background to support the life process of BCAs during transportation and other stress. The oil cakes of TBOs may serve as source of diversified nutrition for BCAs when used as substrate for mass culturing of antagonists.

Materials and Methods

Sources and maintenance of culture

Culture of *Trichoderma harzianum* was isolated from the native soil of University research farm, located at Chiraudi Meerut (UP) India, on *Trichoderma* Selective Medium (TSM). After proper identification and confirmation cultures were maintained on PDA which were stored in refrigerator for further studies.

Screening of different de-oiled cakes and composts for mass multiplication of *Trichoderma harzianum*

Collection of oil cakes and composts: De-oiled cakes of Neem, Jatropha, Mahua and Karanza and two composts (FYM and Vermicompost), used during present investigation, were collected from local agricultural product-processing units and university vermicompost units. These materials were cleaned to remove any unwanted debris such as stones and foreign plant matter. Cakes were crushed in a heavy pestle & mortar to prepare a coarse powder (particles of approximately 1 mm diameter). All de-oiled cakes were mixed with sterilized distilled water (SDW) (10: 2.5, w/v); to maintain 25% moisture (w/v) and placed in conical flasks of 250 ml capacity @100.00 gram/flask (Table 1) followed by autoclaving at 121.6°C (1.1 kg/cm^2) for 20 minutes.

Inoculation of *Trichoderma harzianum* on different substrates: After autoclaving, flasks were allowed to cool at room temperature prior to inoculation. Flasks containing substrates (Table 2) were inoculated

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S. No.	Treatments (De-oiled Cakes)	CFUs of *T. harzianum* × 10^6	
		15 DAI	30 DAI
1.	Neem cake	30.67	42.00
2.	Jatropha cake	27.33	41.67
3.	Mahua cake	26.67	39.33
4.	Karanja cake	24.67	36.33
5.	Farm Yard Manure	12.33	20.00
6.	Vermicompost	13.33	22.33
	CD at 5%	Substrates=1.92 Days=NS Substrates x Days=2.71	

Table 1: Population (CFUs) of *Trichoderma harzianum* on different de-oiled cakes, FYM and vermicompost upto 30 days after incubation.

S. No.	Treatments(De-oiled Cakes)	CFUs of *T. harzianum* × 10^6						
		15 DAI	30 DAI	45 DAI	60 DAI	75 DAI	90 DAI	105 DAI
1.	Neem cake	30.67	42.00	30.33	22.67	17.00	10.33	0.67
2.	Jatropha cake	27.33	41.67	26.33	19.67	16.00	6.67	0.00
3.	Mahua cake	26.67	39.33	23.67	15.67	14.33	5.00	0.00
4.	Karanja cake	24.67	36.33	18.67	15.00	13.67	2.33	0.00
	CD at 5%	Substrates=0.92 Days=1.22 Substrates x Days=2.44						

Table 2: Population (CFUs) of *Trichoderma harzianum* on different de-oiled cakes up to 105 days after incubation.

with 3-4 days old actively growing culture of *T. harzianum* (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 ± 2^0C for 30 days. For each treatment, three replicates of flasks were maintained and arranged in a completely randomized manner. Population dynamics were determined by following serial dilution plate technique.

Determination of longevity and viability of *Trichoderma harzianum* pre-grown on potato dextrose broth medium and latter transferred to different de-oiled cakes: In another experiment, *Trichoderma harzianum* was initially grown on the potato dextrose broth (PDB) medium and then transferred to different de-oiled cakes based substrates. For this purpose, flasks (250 ml Capacity) containing 100 ml PDB were inoculated with five mm disc of *T. harzianum* separately to the respective sets of flasks. The flasks were incubated at 28 ± 2°C for 15 days. After fifteen days of incubation, well grownup culture of fungal antagonist was filtered through Watman No. 42 filter paper and filtrate was discarded, whereas mycelial mat was added to different cakes.

Mixing of mycelial mat with de-oiled cakes: The mycelial mat of *T. harzianum* was collected and mixed to the flasks containing de-oiled cakes of Neem, Jatropha, Mahua and Karanja and incubated at 28 ± 2°C. For this purpose, conical flasks of 250 ml capacity containing 100 g. de-oiled cakes were used. Prior to inoculation, all cakes were mixed with sterilized distilled water (SDW) (10: 2.5, w/v); to maintain 25% moisture (w/v) followed by autoclaving at 121.6°C (1.1 kg/cm^2) for 20 minutes for sterilization. Visual observations on fungal growth were made daily and mixed thoroughly by shaking. Population dynamics were determined at each 15 days interval by following serial dilution plate technique.

Test of antagonistic activity: The *Trichoderma harzianum* original culture and the colonies recovered from deoiled cakes after different duration were tested for their antagonistic activities through dual culture technique against *Sclerotium rolfsii in vitro*.

Results

Screening of different de-oiled cakes and composts for mass multiplication of *Trichoderma harzianum*

The data pertaining to the effect of various substrates on the population dynamics of *Trichoderma harzianum* on different de-oiled cakes have been presented in Table 1. It is evident from the table, that among the substrates tested after 15 days of incubation (DAI), the highest mean population of *T. harzianum* in the form of CFUs (30.67 × 10^6) was supported by Neem cake, which was significantly higher than the mean population (27.33 × 10^6) supported by Jatropha cake. The Jatropha and Mahua (26.67 × 10^6) cakes were found to be at par with each other in supporting the population of *T. harzianum*, while Karanja cake, which supported 24.67 × 10^6 CFUs of *T. harzianum* was lower than the mean population supported by Mahua cake. The vermicompost and FYM which supported 13.33 and 12.33 × 10^6 CFUs of *T. harzianum* were at par with each other but significantly quite lower than the four cakes tested during present investigation.

After 30 days, the highest population of *T. harzianum* was recorded in case of de-oiled cakes of Neem (42.00 × 10^6) followed by de-oiled cakes of Jatropha (41.67 × 10^6) in supporting the population of *T. harzianum,* while de-oiled cakes of Mahua, which supported 39.33 × 10^6 CFUs of *T. harzianum,* was next to deoiled cakes of Jatropha cake. All these values were at par with each other. Deoiled cakes of Karanja was next to Mahua cake which supported 36.33 × 10^6 CFUs of *T. harzianum* and found significantly lower than Mahua cake. Vermicompost which supported 22.33 × 10^6 CFUs was next to the Karanja cake and found significantly lower than all these cakes but higher than FYM. The least population was recorded from FYM (20.00 × 10^6) among all the substrates tested. There was no significant difference between the population obtained after 15 days and 30 days at any substrate.

However, all the four cakes discussed above were further used till 105 DAI to check the longevity and survival of *T. harzianum in vitro.* These data have been presented in Table 2.

After 45 days, it was noticed that highest population of *T. harzianum* was found in Neem cake (30.33 × 10⁶), followed by Jatropha cake (26.33 × 10⁶) which were significantly higher than the Mahua cake, which supported 23.67 × 10⁶ CFUs of *T. harzianum*. The lowest population of *T. harzianum* was supported by Karanja cake (18.67 × 10⁶), which was significantly lower than the mean population found in Neem, Jatropha and Mahua cakes.

At 60 DAI, the highest population of *T. harzianum* was noticed from Neem cake (22.67 × 10⁶), which was significantly higher than the population supported by Jatropha cake (19.67 × 10⁶). The next to Jatropha cake was Mahua cake, which supported 15.67 × 10⁶ CFUs of *T. harzianum.* The lowest performed cake was Karanja, which supported only 15.00 × 10⁶ CFUs of *T. harzianum.* Mahua and Karanja cakes were found to be at par with each other but significantly lower than the mean population of Neem and Jatropha cakes in supporting the population of *T. harzianum.*

The results of 75 DAI, exhibited that highest population of *T. harzianum* was recorded in case of Neem (17.00 × 10⁶) followed by Jatropha (16.00 × 10⁶). The Neem cake was found to be higher than the Jatropha cakes in supporting the population of *T. harzianum,* while Mahua cake, which supported 14.33 × 10⁶ CFUs of *T. harzianum,* was significantly lower than the mean population of *T. harzianum* supported by Jatropha cake. Karanja cake was next and at par to the Mahua cake which supported 13.67 × 10⁶ CFUs of *T. harzianum.*

A significant reduction was recorded in population dynamics at 90 DAI, when highest mean population of *T. harzianum* in the form of CFUs was supported by Neem cake (10.33 × 10⁶), which was significantly higher than the mean population supported by Jatropha cake (6.67 × 10⁶), which were significantly higher than the Mahua cake, which supported 5.00 × 10⁶ CFUs of *T. harzianum*. Least performed cake was Karanja cake which supported 2.33 × 10⁶ CFUs of *T. harzianum* was significantly lower than the mean population of *T. harzianum* on Neem, Jatropha and Mahua cakes.

However at 105 DAI only Neem cake was found to retain the CFUs, which was 0.67 × 10⁶, while other cakes didn't retained any population.

When among all the substrates from 15 to 105 DAIs, values were compared, it was found that highest CFUs were exhibited at 30 DAI that was significantly superior to the values at 15 and 45 DAIs, where CFUs were found statistically at par with each other in case of Neem, Jatropha and Mahua cakes while in Karanja cake, value at 15 DAIs was significantly higher than the value at 45 DAIs. A significant reduction in CFUs was recorded after 60 days and populatin recorded were significantly lower than the population at 45 DAI but significantly higher to the population at 75 DAI. Population of *Trichoderma harzianum* at 75 DAIs were significantly higher than those recorded at 90 DAIs. However, lowest CFUs were noticed after 105 days when most of the substrates lost their ability to support the population dynamics.

When cakes wise comparison was done Neem cake was found to be superior among all the cakes at the entire length of incubation, followed by Jatropha cake. Mahua cake was next to the Jatropha cake in supporting the population of *T. harzianum* and the least CFUs was obtained from Karanja cake during the course of investigation.

Determination of longevity and viability of *T. harzianum* initially grown on potato dextrose broth and transferred to different cakes after 15 days of growth

The results of this experiment (Table 3) indicates that the level of population dynamics was much higher on any of the four cakes when the *T. harzianum* was allowed to grow initially on potato dextrose broth for 15 days and then a 3 g mycelial mat from the broth was added to either of the cakes as compared with when three bits of 5 mm mycelial discs from PDA grown culture were added to these cakes, however the longevity of *T. harzianum* was unchanged. Another interesting point, which was noted was that at 30 days of storage/incubation the population dynamics of *T. harzianum* was comparatively lower (almost significant) than the population dynamics recorded at 15 days. This trend shows that the population dynamics of *T. harzianum* was maximum at 15 days but after 15 days with each advancing 15 days interval the population dynamics showed decreasing trend. It is also worthwhile to mention that Karanja cake could only support the population dynamics up to 75 days and beyond 75 days no population of *T. harzianum* was recorded on Karanja cake, other three cakes were able to support the population of *T. harzianum* up to 90 days Neem cake could support the population up to 105 days also. Interestingly the highest population dynamics of *T. harzianum* was recorded up to 15 days only and at 30 days onward the population showed decreasing trend, whereas in earlier experiments where three bits of 5mm mycelial discs from PDA grown culture were added the increasing trend of population dynamics was noticed up to 30 days and afterward it was declined.

Antagonistic activity

It is worthwhile to mentioned that, the *Trichoderma harzianum* population retrieved from different deoiled cakes after different duration were having sufficient antagonistic activity as it was before growing to different de-oiled cakes.

Discussion

With a purpose to find out a suitable substrates for mass multiplication and also for a longer shelf life of *T. harzianum,* an experiment was conducted to test the suitability of four de-oiled cakes *i.e.,* Neem cake, Jatropha cake, Mahua cake and Karanja cake and also to have a comparison with some substrates already in practice for mass multiplication at a very large scale *i.e.,* farm yard manure (FYM) and Vermicompost. However, the longevity of *Trichoderma* on FYM and Vermicompost was tested up to 30 days only, whereas four de-oiled

S. No.	Treatments (De-oiled Cakes)	CFUs of *T. harzianum* × 10⁶						
		15 DAI	30 DAI	45 DAI	60 DAI	75 DAI	90 DAI	105 DAI
1.	Neem cake	53.67	50.67	42.00	36.33	27.00	13.67	1.33
2.	Jatropha cake	42.67	40.33	33.67	27.00	14.33	3.00	0.00
3.	Mahua cake	37.00	34.67	28.67	22.33	12.33	0.67	0.00
4.	Karanja cake	35.33	32.33	26.00	18.67	8.33	0.00	0.00
	CD at 5%	Substrates=0.84 Days=1.11 Substrates x Days=2.23						

Table 3: Population (CFUs) of Pre grown *Trichoderma harzianum* on Potato Dextrose broth on different de-oiled cakes.

cakes were tested for survival of *Trichoderma* for 105 days. Reason behind testing the vermicompost and FYM for 30 days only, is that, they are all ready known to support the population of *T. harzianum* beyond 30 days, but since in initial trend, the level of population supported by FYM and vermicompost were below the population supported by any of the de-oiled cakes hence FYM and vermicompost were not tested beyond 30 days.

The results indicates that Neem cake supported highest population dynamics of *T. harzianum*. The Neem cake was followed by Jatropha cake with regards to the population dynamics of *T. harzianum*. The Jatropha cake was followed by Mahua cake and it was again followed by Karanja cake. However, differences with regards to the population dynamics among these cakes were not quite great rather there were very small differences but no doubt these were significant. The population dynamics of *T. harzianum* after either 15 or 30 days in the FYM and Vermicompost were quite lower than those recorded in de-oiled cakes hence FYM and vrmicompost were dropped after 30 days. The population dynamics of *T. harzianum* recorded after 30 days in case of either de-oiled cakes or two different composts was quite higher than those recorded after 15 days. It is pertinent to clarify that the population dynamics in two different composts were not monitored after 30 days but in case of four de-oiled cakes *i.e.,* Neem cake, Jatropha cake, Mahua cake and Karanja cake, it was monitored up to 105 days and it was found that up to 30 days trend of population was towards increasing but after 30 days starting from 45 days up to 105 days, the trend of population dynamics of *T. harzianum* was towards decreasing. The population dynamics was monitored after each 15 days and in case of Neem cake the population dynamics at 15 days was lower than the population dynamics recorded at 30 days. However, the population dynamics of *T. harzianum* recorded at 45 days was lower than those recorded at 30 days and after each advancing 15 days, the population dynamics was lower than previous days of monitoring the population dynamics. It was interesting to note that in case of Neem cake there was hardly one or two CFUs $\times 10^6$ at 105 days of incubation/inoculation/monitoring. Rest of the three cakes *i.e.,* Jatropha, Mahua and Karanja cakes also showed the similar trend of supporting population dynamics *i.e.,* up to 30 days it was increasing but after 45 days onward after each advancing 15 days the population dynamics of *T. harzianum* was decreased than previously recorded population dynamics. Unlike Neem cake Jatropha, Mahua and Karanja cakes could support the population dynamics of *T. harzianum* up to 90 days only. At 105 days, no population of *T. harzianum* could be supported by Jatropha, Mahua and Karanja cakes.

The reason behind higher population dynamics of *T. harzianum* on the de-oiled cakes of different Tree Bearing oils (TBO's) may be because of their richness and sufficiency of different type of nutrients, minerals and other constituents which are required and may be supportive for growth and multiplication of *T. harzianum*. Comparatively lower population dynamics of *T. harzianum* on FYM and Vermicompost may be their comparatively less richness with regard to nutrients and other minerals in comparison to de-oiled cakes.

Determination of longevity and viability of *T. harzianum* initially grown on potato dextrose broth transferred to different de-oiled cakes after 15 days of growth

The results of this experiment indicates that the level of population dynamics was much higher on any of the four de-oiled cakes when the *T. harzianum* was allowed to grow initially on potato dextrose broth for 15 days and then a 3 g mycelial mat from the broth was added to either of the cakes as compared with when three bits of 5 mm mycelial discs from PDA grown culture were added to these cakes, however the longevity of *T. harzianum* was unchanged. Another interesting point, which was noted was that at 30 days of storage/incubation the population dynamics of *T. harzianum* was comparatively lower (almost significant) than the population dynamics recorded at 15 days. This trend shows that the population dynamics of *T. harzianum* was maximum at 15 days but after 15 days with each advancing 15 days interval the population dynamics showed decreasing trend. It is also worthwhile to mention that Karanja cake could only support the population dynamics up to 75 days and beyond 75 days no population of *T. harzianum* was recorded on Karanja cake, other three cakes were able to support the population of *T. harzianum* up to 90 days rather Neem cake could support the population up to 105 days also. Interestingly the highest population dynamics of *T. harzianum* was recorded up to 15 days only and at 30 days onward the population showed decreasing trend, whereas in other experiments on any of the cake the increasing trend of population dynamics was noticed up to 30 days and afterward it was declined.

The reason behind this trend may be that in broth culture the *T. harzianum* could have grown so profusely and abundantly that it could have attained its maximum population at its maximum capacity at around 15 days and hence the food sources might have exhaustively exploited and that's why after 15 days the population dynamics may have been started declining.Prior to this study several organic waste, farm waste, agro-industrial byproducts including de-oiled cakes of different TBO's have been tested from time to time by several workers. Jahangirdar et al., reported that among the de-oiled cakes Karanja cake was the best followed by Neem cake and groundnut cake when they were tested for supporting the population dynamics of *T. viride* up to 21 days. During the present investigation, Neem cake was found to be the best than Karanja cake. Jahangirdar et al., had not tested either Mahua cake or Jatropha cake. Sharma and chandel, [3] tested the mass multiplication of three *Trichoderma*spp. *i.e., T. harzianum, T. viride* and *T. virens* on different substrates viz., wheat bran, cotton cake, maize grain, municipal waste, tapioca, saw dust, gladiolus leaves and stems, neem cake, pine needles and *Eucalyptus* spp. and they reported that, all these substrates supported the growth of *Trichoderma* spp. and out of these, wheat bran exhibited maximum CFUs of *T. harzianum* followed by cotton cake, municipal waste and mustard cake. Thus these findings partly support the findings of present investigation.

Mev and Meena, [4] studied the efficacy of solid and liquid forms of wheat straw, shelled maize cob, biogas slurry, saw dust, molasses and sugarcane mud either alone or in combination as substrate for mass multiplication of *T. harzianum* and they reported that *T. harzianum* population increased up to 40 days and then slightly decreased up to 120 days under room temperature [5,6]. These findings are quite in accordance with present findings as during present course of investigation the time interval for population monitoring of *T. harzianum* was 15 days and it was noticed that up to 30 days the population of *T. harzianum* was increased but after 45 days onward it was decreased [7]. To confirm the exact period for which increasing population dynamics trend the time interval for population monitoring should be retested at each 5 days between 30-45 days [8]. Superiority of FYM over different oil cakes for higher population dynamics reported by these workers is not supportive of present findings because during present investigation the oil cakes were found to be superior to FYM and Vermicompost in supporting the population dynamics of *T. harzianum* [9,10]. The reason of differences may be unknown.

In none of the previous studies the Jatropha or Mahua cake have ever been used for mass multiplication of *T. harzianum* although, Neem and Karanja cakes have so far been tested for such purpose by several workors [11]. It can be interpreted that nutrition rich compounds such as N, P, K, some of the minerals and vitamins and lipids available in de-oiled cakes might be supporting the higher population dynamics of *T. harzianum* over a longer period [12]. Comparatively lower population dynamics retrieved from FYM and Vermicomposting as compared to de-oiled cakes may be attributed to their comparatively less nutritional status than the de-oiled cakes. The reason behind decline of population dynamics after 30 days during present investigation may be that, at the initial level there may be plenty of nutrition supply available for growth and sporulation of *T. harzianum* which later get declined, as they might have been exhausted day by day due to utilization by growing *Trichoderma* in the substrate itself and resulted in poor supply and thereby lower population dynamics with prolonging duration of storage [13-15].

Conclusion

Based on these findings it can be concluded that any of these de-oiled cakes *viz* Neem(*Azadirachta indica*), Mahua(*Madhuca longiifolia*),Jatropha (*Jatropha curcas*) and Karanja (*Pongamia pinnata*) can be readily used for mass multiplication of *Trichoderma harzianum* with a very high level of population dynamics of fungal antagonist. Utilization of de-oiled cakes as substrate for mass multiplication will help in enhancing fertility status of field soil in addition to minimizing the risks of disease occurrence.

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