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Biocontrol and Homeopathic Approaches Against *Neopestalotiopsis protearum*: A Novel Strategy for Managing Sapota Leaf Spot

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ABSTRACT

Samples of leaf spot disease were collected from a sapota orchard on the university campus and brought to the laboratory. The pathogen was isolated using the hyphal tip technique, and its pathogenicity was confirmed in both pot culture and moist blotter paper method. Neopestalotiopsis protearum isolate was identified based on cultural characteristics, morphology, pathogenicity tests, and molecular analysis. In the present investigation, biocontrol agents and homeopathic medications are used to combat the sapota leaf spot pathogen. Isolation, screening, and field testing of several biocontrol/homeopathic remedies requires substantial time, people, and resources. A total of 3 fungal and 2 bacterial bioagents were evaluated against N. protearum usingdual culture technique, out of which Trichoderma asperelloides and Trichoderma asperellum were found most effective which recorded least mycelial growth of 2.25 cm, and 2.88 cm corresponding to highest mycelial growth inhibition 74.31% and 67.14% respectively of the test pathogen over untreated control followed by Pseudomonas fluorescens and Trichoderma harzianum which recorded 3.38 cm and 3.70 cm radial mycelial growth and 61.43% and 57.71% inhibition of the test pathogen respectively. Ten homeopathic drugs at the concentration of 100 ppm were evaluated by employing poisoned food technique against N. protearum out of which Arsenicum and Rhus toxicodendrum had the highest mycelial growth inhibition percentage (100%), which was noteworthy when compared to the other drugs tested.

Keywords: *Antagonists, biocontrol, homeopathic drugs, inhibition, bioagents, leaf spot, sapota, Neopestalotiopsis protearum.*

Introduction

Sapota, also known as chiku (*Achras zapota* L.), is primarily grown in India for its fruit, while in South-East Mexico, Guatemala, and other countries it is cultivated for the production of chickle, a gum-like material derived from latex, commonly used in making chewing gum. The fruit contains a high amount of easily digestible sugar (15-20%), as well as a notable amount of proteins, fats, fibers, and minerals such as calcium, phosphorus, and iron. Sapota pulp is utilized in the preparation of candies and halwas. It is used as a component in fruit salads and milk-shakes. Sapota is predominantly cultivated in the states of Gujarat, Maharashtra, Karnataka, Tamil Nadu, Andhra Pradesh, and Kerala in India. Gujarat is the top producer of spaota, with a share of 32.84%, followed by Andhra Pradesh at 24.24% and Maharashtra at 13.08%. *Neopestalotiopsis* species (formerly known as *Pestalopsis*) are plant pathogens that cause various diseases in Sapota plants such as canker lesions, shoot dieback, needle blight, tip blight, scabby canker, leaf spots, grey blights, leaf blights, and fruit rot, leading to significant economic loss in certain situations.

Neopestalotiopsis leaf spot is characterized by the presence of many small, circular, pinkish to reddish brown spots on mature leaves, with a visible white center and a water-soaked appearance. Spots combine and leaves fall prematurely. This leads to a decrease in the transportation of nutrients to the fruits, resulting in a decrease in their size. Spots cover the whole fruit within 3 to 4 days on fruits. Fruits turn mushy and dark brown, with many acervuli appearing in the decayed areas afterwards.

Chemical fungicides are still widely used to manage most diseases, including leaf Sapota leaf spot, and also observed promising results of their application. However, their use has resulted in the accumulation of toxic chemicals that are potentially dangerous to humans and wildlife. As a result, the utilization of microorganisms native to the same environmental conditions and certain homeopathic drugs should be useful in controlling pathogen growth or reducing its population to an appropriate level. Bacteria and filamentous fungus regarded to be promising biocontrol agents biocontrol agents with broad-spectrum antifungal properties. Biocontrol agents like *Trichoderma* species, *Bacillus subtilis* and *Pseudomonas fluorescens* have effectively inhibited the spread of the pathogen on fruits [4] [20].

Homeopathic remedies have demonstrated effectiveness against various phytopathogenic fungi in several economically important crops. These include *Phytophthora parasitica* var. *piperina* in betelvine [7], *Colletotrichum gloeosporioides* in mango [3], *Penicillium expansum* in pear fruit, *Alternaria solani* in tomato [19], and *Ascochyta rabiei, Phomopsis vexans,* and *Fusarium oxysporum* f. sp. *lycopersici* in chickpea, brinjal, and tomato, respectively [9]. Therefore, homeopathic treatments could serve as a potential strategy for mitigating the occurrence of diseases in sapota crop.

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Material and Methods

The current study was conducted at the Department of Plant Pathology and AICRP on Fruits scheme laboratory, Dr. PDKV, Akola.

Source of bioagents

Bioagents, viz. *Trichoderma asperelloides, Trichoderma asperellum, Trichoderma harzianum, Bacillus subtillis* and *Pseudomonas fluorescens*e were procured from Centre for Organic Agriculture Research and Training, Dr. PDKV, Akola.

Source of Homeopathic drugs

The homeopathic drugs used in this study *viz.* Sulphur, Silicea, Sepia, Arsenicum, Kali iodatum, Graphite, Catcarea carbanica, Mercurians solubilis, Natrum muriaticum and Rhus toxicodendrum, were purchased from the specialized Raja Homeopathic Pharmacy, Akola at a dynamization of 30CH (Centesimal Hahnemannian).

Evaluation of bioagents under laboratory conditions

The dual culture technique [9] was used to test the antifungal activity of bioagents. The different bioagents (*Trichoderma asperelloides, Trichoderma asperellum, Trichoderma harzianum, Bacillus subtillis,* and *Pseudomonas fluorescens*) were tested against test pathogen. The test pathogen was placed (6 mm) in the centre of the media, and the fungal test bioagents were cut out with sterilized cork borer and placed at equidistance exactly opposite to the test pathogen on autoclaved and cooled solid surface of PDA medium in Petri plates. The plates were then incubated at 25 ± 2°C. PDA plates inoculated alone with pure culture disc of the test pathogen were maintained as control. For the bacterial antagonists, streaking was done in parallel line on both the sides of the pathogen discs (at a distance of 15 mm) kept in the center. Plates inoculated with pathogen alone served as control. The experiment was conducted in Completely Randomized Design (CRD) with six treatments and four replications. The radius of the pathogen colony was measured after 7 days of incubation and per cent inhibition, the percentage inhibition of the pathogen by the antagonist was calculated by applying formula suggested by Vincent [26].

In vitro evaluation of homeopathic drugs by poisoned food technique

Ten homeopathic drugs were evaluated by employing poisoned food technique [19] against *Neopestalotiopsis protearum.*

The efficacy of different homeopathic drugs (Sulphur, Silicea, Sepia, Arsenicum, Kali iodatum, Graphite, Catcarea carbanica, Mercurians solubilis, Natrum muriaticum and Rhus toxicodendrum) at concentrations of 100 ppm (100 µl/ L) was evaluated in PDA medium using poisoned food technique against test pathogen in the laboratory accordingly to the methodology described by Kumar [16] (Table 1). The test pathogen was grown on PDA medium for 12 days before setting up the experiment. The PDA medium was prepared and melted, and homeopathic medicine was added to the melted medium at the required dynamizations concentrations. Twenty ml of poisoned medium was poured into sterilized Petri-plates. The suitable check was maintained without the addition of homeopathic medicine (control). A mycelial disc of 6 mm was taken from the periphery of the young sporulating colony. The actively growing hyphal tip was removed by cork borer and placed in the center of poisoned Petri plates incubated at 25 ± 2°C until the control plate was full.

The radial growth of the fungus on the poisoned medium was measured on a daily basis up to 8 days of inoculation. The diameter of the colony was measured in two directions and the average was recorded to find out the growth of concerning pathogens. Experiment was laid out in Completely Randomized Design with 12 treatments (10 homeopathic medicine, one standard fungicide and control treatment) in triplicate and the per cent reduction of mycelial growth over control was calculated using the following formula of Vincent [26].

Table 1. Homeopathic drugs used in experimentation

Treatment	Homeopathic drugs	Concentration used
T_1	Sulphur	100 ppm
T_2	Silicea	100 ppm
T_3	Sepia	100 ppm
T_4	Arsenicum	100 ppm
T_5	Kali iodatum	100 ppm
T_6	Graphite	100 ppm
T_7	Catcarea carbanica	100 ppm
T_8	Mercurians solubilis	100 ppm
T_9	Natrum muriaticum	100 ppm
T_{10}	Rhus toxicodendrum	100 ppm
T_{11}	Mancozeb 50% + Thiophanate Methyl 25 % WG	0.25%
T_{12}	Control	-

Result and Discussion

In vitro evaluation of bio-agents on growth inhibition of *N. protearum*

In the present study, total five bio-agents were evaluated *in vitro* against *N. protearum* by applying dual culture technique and per cent inhibition of the respective pathogen over control was calculated and presented in Table 2 and Plate 1.

Three fungal (*Trichoderma asperlloid, Trichoderma harzianum* and *Trichoderma asperallum*) and two bacterial (*Pseudomonas fluorescens* and *Bacillus subtilis*) bio-agents were evaluated *in vitro* against *N. protearum*. Results revealed that all the bio-agents evaluated exhibited fungistatic activity and significantly inhibited mycelial growth of *N. protearum* (Table 2). Of the five bio-agents tested, *Trichoderma asperelloides* and *Trichoderma asperellum* were found most effective which recorded least mycelial growth of 2.25 cm, and 2.88 cm corresponding highest mycelial growth inhibition 74.31%, and 67.14% respectively of the test pathogen over untreated control. *Pseudomonas fluorescens* and *Trichoderma harzianum* were found next effective with 3.38 cm and 3.70 cm radial mycelial growth and 61.43% and 57.71% inhibition of the test pathogen respectively. Maximum growth of *N. protearum* was observed in control plate i.e. 9.00.

Table 2. Effect of bioagents on radial mycelial growth of N. protearum (7 DAI)

S.N	Bioagents used	Radial mycelium growth (cm)	% Growth inhibition
1	*Trichoderma asperelloides*	2.25	74.31
2	*Trichoderma asperellum*	2.88	67.14
3	*Trichoderma harzianum*	3.70	57.71
4	*Bacillus subtilis*	5.59	36.11
5	*Pseudomonas fluorescens*	3.38	61.43
6	Control	8.75	-
	SE m (±)	0.19	-
	CD (P=0.01)	0.79	-

Excessive pesticide uses in fruit crops, as well as reliance on them, will result in problems of poisoning, environmental hazards, residual effects, and resistance development. Therefore, there is a need for more rational fungicide use, as well

as the identification and implementation of successful alternative approaches. The purpose of this study was to compare the efficacy of three fungal biocontrol agents, *Trichoderma asperelloides, Trichoderma asperellum, Trichoderma harzianum* as well as two bacterial biocontrol agents (*Bacillus subtilis and Pseudomonas fluorescens*) against *N. protearum*. Because of their unique antagonistic properties, both fungal and bacterial bio-control agents significantly reduced *N. protearum* growth. However, fungal antagonists were more effective than bacterial antagonists.

Current study shows that *Trichoderma* species successfully control the fungal pathogen. *Trichoderma* support plant growth and combat diseases by myco-parasitism, antibiosis, environmental modification, and stimulation of plant defense mechanisms. In addition to many other functions, *Trichoderma* competes with pathogens for nutrients and space [1]. The zone of inhibition seen in the dual culture technique could be attributed to the antibiosis phenomenon. Extracellular enzyme production, such as exochitinases, may have resulted in the creation of such a clear zone [15] [7]. These enzymes dissolved host cell fragments, causing the creation of more enzymes and triggering a cascade of physiological changes that stimulated *Trichoderma* species rapid and directed growth [27]. When in contact with a pathogen, one of the recognized pathways in pathogen antagonism is the production of cell wall lytic enzymes such as chitinase, glucanase, and protease, as well as metabolites such as harzianic acid, alamethicins, tricholin, and peptidaibols [25] [23] [4]. The findings are also consistent with those of Kumhar [17], who found that *Trichoderma* inhibited *Pestalotiopsis theae* mycelial growth at a maximum of 61.50%, which is consistent with the results of this investigation. Amrutha and Vijayaraghavan [2] found that *Trichoderma asperellum* repressed the *Neopestalotiopsis clavispora* pathogen by 66.67%, whilst *Pseudomonas fluorescens* inhibited it by 56.67%, supporting the current findings.

The current investigation indicated that bacterial antagonists were significantly less effective as compared to the fungal antagonists. Majority of fungal cell walls are made of chitin. Most fluorescent pseudomonads and *Bacillus* are weak makers of the chitinase enzyme [24], this might be the reason the tested bacterial biocontrol agents had limited antagonistic potential. Barman *et al.* [4] found that *P. fluorescens* had a limited antagonistic potential against *Pestalotiopsis theae* when tested in laboratory conditions.

Bacillus subtilis antagonist was found to be less effective compared to tested antagonists. On contrary, Saju *et al.* [23] reported that *B. subtilis* inhibited *Pestalotiopsis* sp. the most (62.6%), followed by *T. viride* (50.9%). Zhang *et al*. [28] also found that *Bacillus* strains showed substantial antagonistic effects in the dual culture test, with inhibition rates ranging from 50.22% to 79.48% against *Neopestalotiopsis clavispora*. Contradictory results could be attributed to *Bacillus* strain differentiation in the present investigation, a different pathogen investigated, or laboratory condtions.

Biocontrol agents substantially suppressed *Neopestalotiopsis protearum* growth *in vitro*. *In vivo*, environmental factors and competition with other microorganisms can restrict the efficacy of antagonist fungi with high bio-control levels. Biocontrol potential in field environments requires further investigation.

In vitro evaluation of homeopathic drugs on growth inhibition of *N. protearum*

Homeopathy is currently being employed in a variety of agricultural applications, including insect control and plant disease treatment. It increases active principles or secondary metabolism in plants, influencing plant physiology. Homeopathy can help plants maintain their health by its tonic impact and/or direct inhibition of pathogenic agents. Hence, in the present study, total ten homeopathic drugs were evaluated *in vitro* against *N. protearum* by applying poisoned food technique at concentrations of 100 ppm and per cent inhibition of the pathogen over control was calculated. For comparison purposes, both standard fungicide control and absolute control were maintained.

Results showed a significant inhibition of the mycelial growth of *N. protearum* in all ten tested concentrations over control (Table 3 and Plate 2).

Table 3. Effect of homeopathic drugs on radial mycelial growth of N. protearum (7 DAI)

Treatment	Homeopathic drugs	Conc. used	Radial mycelial growth (cm)	% Growth inhibition
T1	Sulphur	100ppm	5.63	37.44
T2	Silicea	100ppm	6.93	23.00
T3	Sepia	100ppm	8.0	11.11
T4	Arsenicum	100ppm	0.00	100.0
T5	Kali iodatum	100ppm	7.14	20.66
T6	Graphite	100ppm	1.10	87.77
T7	Catcarea carbanica	100ppm	6.77	24.88
T8	Mercurians solubilis	100ppm	3.90	56.66
T9	Natrum muriaticum	100ppm	8.97	0.44
T10	Rhus toxicodendrum	100ppm	0.00	100.0
T11	Mancozeb 50% + Thiophanate Methyl 25 % WG	0.25%	0.00	100
T12	Control	_	9.0	_
	SE m (±)	_	0.08	_
	CD (P=0.01)	_	0.32	_

Arsenicum and Rhus toxicodendrum had the highest mycelial growth inhibition percentage (100%), which was noteworthy when compared to the other drugs. Fungicide control: Mancozeb 50% + Thiophanate Methyl 25% WG @ 0.25% showed 100% growth suppression of *N. protearum*. The next best homeopathic medication was graphite, which inhibited growth by 87.77%, followed by Mercurians solubilis (56.66%). The remaining medications inhibited growth by less than 50%. Natrum muriaticum showed the least percentage of growth suppression (0.44%), followed by Sepia (11.11%) (Plate 2).

Homeopathic medicines are less expensive and have no negative effects, so effort was made to evaluate their effect on *N. protearum in vitro*. Among the drugs tested, Arsenicum and Rhus toxicodendrum were most effective, inhibiting mycelial growth 100%, followed by Graphite (87.77%). Researchers have reported the Homeopathic drugs in controlling plant pathogens. Khanna and Chandra [12] reported that fungitoxicity of Homoeopathic drugs such as Arsenicum album, Kali iiodatum, Lycopodium clavatum, Phosphorous, Thuja occidentalis, Asvagandh, Blatta orientalis, Zincum sulphuricum, Flix mas, and Kali muriaticum against *Pestalotia mangiferae*. Homeopathic drugs have been shown to effectively control various phytopathogenic fungi in important crops, including *Aspergillus niger* in coriander and cumin [18], *Botryodiplodia* in guava fruit [11], betel vine disease caused by *Phytophthora parasitica* var. *piperina* [8]. In addition, Patil and Suryawanshi [22] reported that when combined with mancozeb, sulphur 30 CH and Nux vomica has the highest percentage control efficacy of *Alternaria alternata* (84.45%), followed by Cina, Rhus toxicodendron, Arnica montana, Sanguinaria canadensis, Tarentula hispana, and Selenium. In the case of *C. gloeosporioides*, Ashraful *et al.* [3] reported that Arsenicum album significantly outperformed over Selenium, Nux vomica, Belladonna, and Calcarea fluorica. Homeopathic drugs, such as Lycopodium spp., Thuja spp., Arsenicum spp., and Zincum spp., have been shown to be effective in controlling fruit rot pathogens such as *F. moniliforme, A. alternata, Gloeosporium psidii, Colletotrichum gloeosporioides*, and *Pestalotia* spp. [13]. Furthermore, Pallavi Mahajan *et al.* [21] found that Sulphur, Pulsatila nigricans, Podophyllum pellatum, Cina, Lycopodium clavatum, Nux vomica, Dulcamara, Colocynthis, Aconitum napellun, Natrum muriatum, Rhux toxicodendron, Arnica montana, Hepar sulphur and Arsenicum album completely inhibited *Alternaria solani* growth. The drugs like Apis melifera, Cinchona officinalis, Sepia and Calcaria carbonica suppressed *Alternaria solani* by 87.78, 80.00, 64.44 and 57.78%, respectively.

Homeopathic medicines may inhibit *N. protearum* due to the presence of various metabolites, including phenolics, polyphenols, tannins, quercetin, flavonoids, alkaloids, terpenoids, volatile oils, polypeptides, and complex mixtures in their mother tinctures. This study does not examine the active chemicals in homoeopathic formulations or how they suppress the growth of plant pathogenic fungi *N. protearum in vitro*.

Conclusion and future scope

In present study fungal antagonists *Trichoderma asperelloides, Trichoderma asperallum*, and bacterial antagonist *Pseudomonas fluorescens* outperformed other bio-agents in inhibiting *Neopestalotiopsis protearum* growth. Arsenicum and Rhus toxicodendrum outperformed other homeopathic drugs in preventing *N. protearum* growth. The integration of biocontrol and homeopathic approaches for managing *Neopestalotiopsis protearum*, the causative organism of sapota leaf spot, offers a potential and environmentally sustainable alternative to traditional chemical management. Homeopathic drugs have the potential to replace synthetic products and promote sustainable and eco-friendly agriculture. However, further study is required for dose standardization and practical implementation. Large-scale, multi-location field experiments are required to test the efficacy and reliability of these approaches under variable agro-climatic conditions.

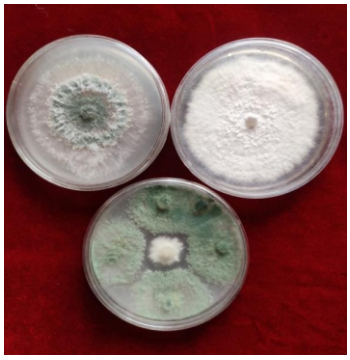
Acknowledgements

The authors sincerely thank the Head, Department of Plant Pathology, Dr. PDKV, Akola and Project Coordinator (Fruits), Bengaluru, for providing necessary facilities.

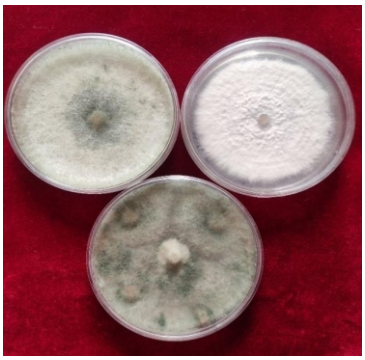
Conflicts of Interest

The authors declare no conflict of interest.

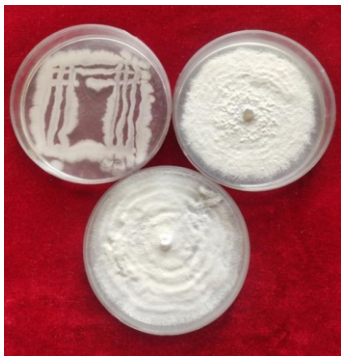
T1 (Trichoderma asperelloides)



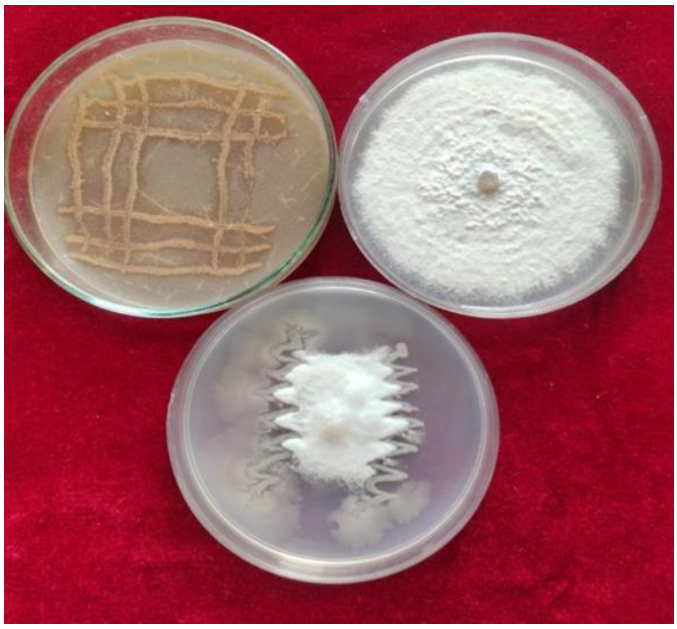
T2 (Trichoderma asperellum)



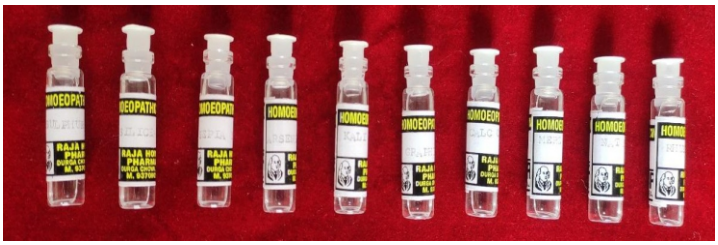
T3 (Trichoderma harzianum)



T4 (Bacillus subtillis)



T5 (Pseudomonas fluorescens)



Homeopathic drugs

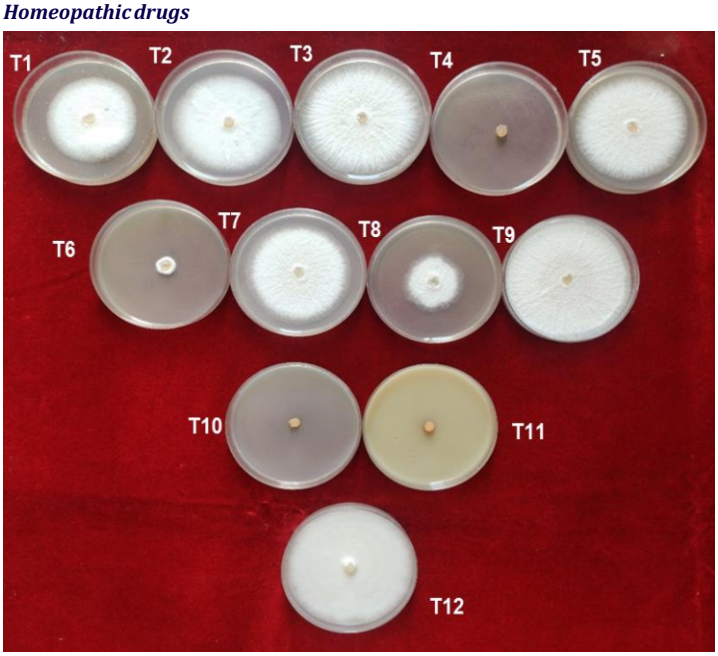


Plate 2. Effect of homeopathic drugs on radial mycelial growth of N. protearum

Table 1. Homeopathic drugs used in experimentation

Treatment	Homeopathic drugs	Concentarion used
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T2	Silicea	100 ppm
T3	Sepia	100 ppm
T4	Arsenicum	100 ppm
T5	Kali iodatum	100 ppm
T6	Graphite	100 ppm
T7	Catcarea carbanica	100 ppm
T8	Mercurians solubilis	100 ppm
T9	Natrum muriaticum	100 ppm
T10	Rhus toxicodendrum	100 ppm
T11	Mancozeb 50% + Thiophanate Methyl 25 % WG	0.25%
T12	Control	-

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6	Control	8.75	_
	Test	Sig	_
	SE m (±)	0.19	_
	CD (P=0.01)	0.79	_

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T9	Natrum muriaticum	100ppm	8.97	0.44
T10	Rhus toxicodendrum	100ppm	0.00	100.0
T11	Mancozeb 50% + Thiophanate Methyl 25 % WG	0.25%	0.00	100
T12	Control	_	9.0	_
	Test	_	Sig	_
	SE m (±)	_	0.08	_
	CD (P=0.01)	_	0.32	_

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