The Journal of Animal & Plant Sciences, 27(4): 2017, Page: 1321-1330
ISSN: 1018-7081

EVALUATION OF CARRIER MATERIALS TO DEVELOP *BACILLUS SUBTILIS* FORMULATION TO CONTROL ROOT KNOT NEMATODE INFECTION AND PROMOTE AGROECONOMIC TRAITS IN EGGPLANT

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ABSTRACT

Preparation of inducer inoculum in the most suitable carrier material is imperative to get full benefit from inducer species under field conditions. Present study mainly focuses upon the evaluation of supportive behavior of carrier materials (i.e. sand, saw-dust, bagasse, talc and bentonite) toward *Bacillus subtilis* in controlling *Meloidogyne incognita*, Root Knot Nematode (RKN) of brinjal crop. Inoculum of bacterial inducer was prepared in five different carriers and incubated at room temperature for 0, 10, 20…80 days. Then, Field applications of the treatment revealed that bagasse was the best carrier material, which not only controlled the disease but also enhanced the growth of brinjal plants. Furthermore, the second most efficient carrier was saw-dust, while efficiency was gradually descended in an order, talc > sand > bentonite. Although bagasse showed maximum efficacy in term of disease control and growth promotion of plants. However, it did not support the number leaf hair leaving the plant vulnerable to foliar insect pest. Saw-dust based formulations are recommended for the areas where insect attack is most common on foliar parts of the crop because it enhanced the number of leaf hair significantly. Current investigation is a contribution in managing nematode plant diseases using environmentally safe 'biocontrol agents'. Present study is a major contribution in plant disease control program and might be a significant plant protection strategy in future.

Keywords: Root knot nematode, Agroeconomic traits, Disease severity, Inducer, Innate resistance.

INTRODUCTION

Controlling plant pathogens using biotic inducers is a preferred technique due to its effective control, the lowest cost and environment friendly behavior (Pal and Gardener, 2006).*B. subtilis* is an efficient resistance inducer and its field formulations are needed to be prepared in carrier materials in order to make its handling easier and more effective. There is also a chance that use of carrier materials may greatly affect the expected results (Espinel-Ingroff *et al.,* 2004). Application of an inoculum in field always required a suitable carrier material (Rabea and Steurbaut, 2010). Use of the carrier material for inoculum application is necessary as it make their handling easier and provide them sufficient nutrition to retain their efficiency. Therefore, carrier medium used for the application of biocontrol agent express very positive effect on the crops (Espinel-Ingroff *et al.,* 2004). Furthermore, a suitable carrier essentially support inducers for a significant time period and preserve preferred characteristics of inducer integral (Nakkeeran *et al.,* 2005). The formulation is considered as a reliable source for resistance induction and disease management of a cultivar, if the carrier (in which formulation is prepared) also bear all above mentioned characteristics. Therefore, it's imperative to screen out the best carrier material to get the maximum advantage from resistance inducer.

Currently, potential of different carrier materials was investigated to support disease control and growth promoting behavior of *B. subtilis*. The study has been performed on *Meloidogyne incognita*, causing root knots in brinjal. It will help out researchers to prepare commercial inoculum of *B. Subtilis* to control plant parasitic nematode.

MATERIALS AND METHODS

Preliminary studies screened two brinjal cultivars, one was resistant and the other was susceptible to plant parasitic nematodes (i.e. Nirala = Resistant; Dilnasheen = Susceptible, against *M. incognita*).Seeds of the representative cultivars, culture of inducer species (*B. subtilis*) and culture of pathogen (*M. incognita*) were procured from 'Plant Nematology Lab., University of the Punjab, Lahore, Pakistan'. Five different carrier materials i.e. Talc, Bentonite, Sand, Saw-dust and Bagasse were purchased from 'Medinnov Corp. Firdous Market Gulberg-III, Lahore, Pakistan' to perform the study.

Inoculum of *B. subtilis* was prepared by inoculating100 g of each carrier with inducer inoculum in such a way that initially concentration of spores was maintained 1×10^5 spores/ g of dried inoculum (Shazia *et al.,* 2015). Inocula (100 g packets) were prepared with the difference of ten consecutive days (i.e. 0, 10, 20, 30…80 days). Prepared inocula were stored at room temperature. Seeds of brinjal cultivars were grown in experimental field of Institute of Agricultural Sciences, University of the Punjab, Lahore. Soil used to grow brinjal seeds was sandy loam with pH 7.2. At the age of one month, plants were divided into sets in which each cultivar exhibited equal share; while each set consisted upon ten replicates (500 plants in each replicate).Sets of single cultivar were further distributed into five groups (10 sets in each group); leaving one set as 'pathogen control (PC)'. Nine sets of each group were treated with *Bacillus* inoculum of variable incubation periods, leaving one set as carrier control. Inducer inoculum treatments prepared in different carrier materials (5 g/plant) were applied in soil just before a scheduled watering session. However, pathogen inoculum (5 mL, 300 eggs/mL) was applied to each plant, letting PC for pathogen inoculum only. After two weeks data were recorded regarding disease severity (DS) on the basis of percentage of infected root. Percentage disease control (DC) was calculated through following formula.

$$\text{Percentage control} = \frac{\text{DS of PC} - \text{DS of carrier treatment}}{\text{DS of PC}} \times 100$$

Data were statistically analyzed using DSAASTAT (Onofri, Italy). Plants were watered up to three months of age and then data were again recorded regarding agro-economic traits i.e. plant height, stem diameter (at 2" height from soil surface), number of leaves per plant, number of fruits per plant, number of branches, average weight of five fruits, chlorophyll contents and number of leaf hairs per unit leaf area. Chlorophyll contents were measured in g/kg by adopting the method of Bojović and Stojanović (2005). Efficiency of all carrier materials was calculated after measuring agro-economic traits and the best carrier material was evaluated according to Shazia *et al.,* (2015).Moreover, correlations among carrier materials, inducers, DS and agroeconomic traits were also determined by calculating homoscedasticity. Thus, homoscedastic values were calculated for Pearson's Correlation Coefficient (PCC) using R Statistical Package (Gentleman R and Ihaka R, Aukland, New Zealand), and values were plotted across correlation figures. Experiment was independently repeated twice to ensure reliability of the results.

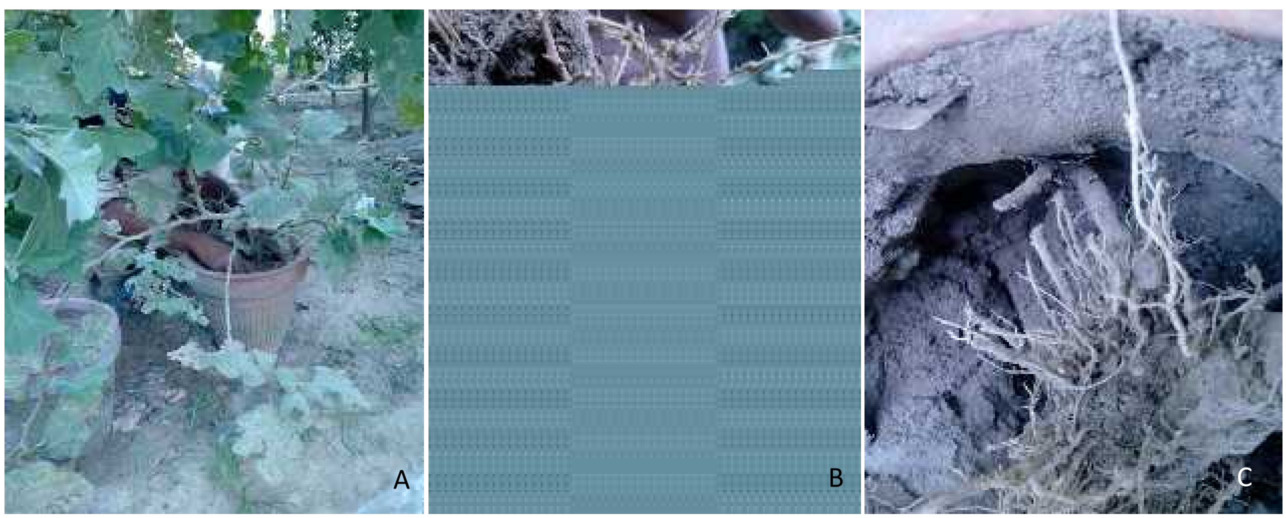


Figure 1. (A) and (B).Inoculum applied without carrier material; (C). Roots of the eggplant treated with *B. subtilis* in bagasse.

RESULTS

Maximum DS (13.61%) was recorded on Dilnasheen in case of sand based treatment proving it the least suitable carrier material for formulation preparation. Its DS was more than double of the pathogen control treatment of Nirala. The least DS was recorded by bagasse based formulation (0.79% on Dilnasheen and 0.09% on Nirala) declaring it the best suitable option for preparation of formulation. Saw-dust closely followed the bagasse with 1.66% and 0.6% DS on Dilnasheen and Nirala, respectively. Talc formulation was the least supportive toward agroeconomic traits of brinjal plants in terms of plant height. However, bagasse and saw-dust formulations were more or less equally supportive toward plant height. Bagasse showed more positive behavior toward all agronomic traits of brinjal plants except leaf hairs. Number of leaf hairs was denser due to application of saw-dust formulation than the application of bagasse formulation (Table 1).Carrier control treatments of rest of the carrier materials did not show any relation with disease control.

Table 1. Formulation of *B. subtilis* prepared in different carrier materials were tested for disease severity and other agroeconomic traits

		Talc				Bentonite				Sand				Sawdust				Bhagasse				Control		
		Talc-F	P+C	I+C	CC	Bent-F	P+C	I+C	CC	Sand-F	P+C	I+C	CC	SwD-F	P+C	I+C	CC	Bha-F	P+C	I+C	CC	PC	IC	NC
Disease Severity	N	2.49	6.00	0.00	5.81	2.73	6	0.00	6	4.44	6.00	0.00	6	0.6	6	0.00	6	0.09	6	0.00	6	18.09	6	0.1
	D	7.95	18.0	0.1	17.5	9.08	18	0.1	18	13.6	18	0.1	18	1.66	17.9	0.1	17.9	0.79	17.8	0.1	17.9	6	18.7	0.81
Plant Height (cm)	N	66	48.5	104	50.4	67.7	48.5	104	51.1	80.3	48.5	104	56.3	95.3	48.5	105	56.2	104	48.5	105	53.1	50.1	105	53.3
	D	62.6	40.3	111	50.7	67	40.3	111	50.1	74.6	40.3	111	56.1	96.3	40.3	111	54.1	100	40.3	111	52.2	40.2	101	52.9
Stem diameter(cm)	N	1.25	0.94	1.88	0.95	1.36	0.94	1.88	0.9	1.52	0.94	1.88	0.91	1.71	0.94	1.88	0.91	1.81	0.94	1.88	0.91	1	1.83	0.96
	D	1.27	0.91	1.81	0.96	1.30	0.91	1.81	0.9	1.50	0.91	1.81	0.91	1.69	0.91	1.81	0.9	1.83	0.91	1.81	0.9	0.98	1.82	0.91
No. Leaves/Plant	N	129	82.4	189	97.2	124	82.4	189	79.1	126	82.4	189	76.1	181	82.4	189	76.1	190	82.4	189	71.1	81.9	191	71.5
	D	122	82.7	188	99.1	117	82.7	188	71	134	82.7	188	76	177	82.7	188	73	185	82.7	188	75	81.4	187	75.1
No. Fruits/ Plant	N	75.4	45.7	118	47.2	73.8	45.7	118	32.4	57.8	45.7	118	30.1	114	45.7	118	34.1	121	45.7	118	30	45.8	122	30.5
	D	75.6	41.6	119	49.2	76.5	41.6	119	31.1	58.2	41.6	119	33.2	106	41.6	119	31.2	119	41.6	119	29.2	40.6	120	29.5
No. Branches/Plant	N	23.4	17.4	17.9	18.1	23.4	17.4	17.9	15.3	19.8	17.4	17.9	15.1	29.7	17.4	17.9	16.1	34.2	17.4	17.9	15.1	17.5	34.6	15.5
	D	23.5	17.8	17.4	19.1	22.2	17.8	17.4	16	19.8	17.8	17.4	15	30	17.8	17.4	16	34.2	17.8	17.4	16	17.1	34.3	16.2
Weight of 5 Fruits (g)	N	170	84.2	83.9	102	176	84.2	83.9	51.1	164	84.2	83.9	49.1	186	84.2	83.9	53	194	84.2	83.9	55.1	83.9	196	55.7
	D	171	61.7	81.2	93.1	164	61.7	81.2	52.2	150	61.7	81.2	43	175	61.7	81.2	50.2	186	61.7	81.2	56.1	58.4	189	56.1
Chlorophyll Content (g/kg)	N	0.94	0.69	0.69	0.72	0.79	0.69	0.69	0.31	0.66	0.69	0.69	0.28	1.18	0.69	0.69	0.31	1.26	0.69	0.69	0.33	0.67	1.29	0.38
	D	0.89	0.48	0.51	0.69	0.71	0.48	0.51	0.33	0.6	0.48	0.51	0.29	1.16	0.48	0.51	0.28	1.28	0.48	0.51	0.31	0.43	1.28	0.34
Leaf Hair	N	12.5	3.04	3.73	3.11	11.4	3.04	3.73	5.01	9.22	3.04	3.73	4.03	24.1	3.04	3.73	4.2	23.5	3.04	3.73	3.12	3.01	23.9	3.19
	D	13.1	3.05	3.65	4.14	10.4	3.05	3.65	3.03	8.55	3.05	3.65	3.1	23.8	3.05	3.65	4.11	23.5	3.05	3.65	4.03	3.02	23.6	4.08

Formulations have been mentioned with short names as, (Talc-F) talc formulation, (Bent-F) bentonite formulation, (Sand-F) sand formulation, (SwD-F) saw-dust formulation, Bha-F) bagasse formulation. Control treatments included in this study were (P+C) pathogen + carrier, (I+C) inducer + carrier, (CC) carrier control, (PC) pathogen control, (IC) inducer control and (NC) negative control; while representation of resistant and brinjal cultivars was made by (N) Nirala and (D) Dilnasheen.

Observed agronomic traits include (i.e. plant height (cm), stem diameter (cm), number of leaves, number of fruits, number of branches, weight of fruits (g), chlorophyll contents (g/kg) and leaf hairs.

Maximum percentage DC was shown by bagasse and saw-dust inocula of zero day incubation period (Figure 2).Bagasse supported the resistance induction potential of *B. subtilis* for longer durations than saw-dust, because after 80 days of incubation bagasse supported approximately 90% DC activity in comparison with 80% DC of saw-dust. Sand was really a good carrier only at 0 days of incubation, drastically reducing its efficacy after 10 days of incubation. Its efficacy was sharply decreased from 0-50 incubation days. More incubation days did not cause further decrease in percentage DS (Figure 2). Bentonite and Talc were not better carriers than sand for short incubations and provided lesser DC also. After 50 days of incubation, Talc and Bentonite showed 50% and 37% average DC, respectively, which was significantly greater than 0% DC of respective sand inoculum. Talc was relatively better carrier than Bentonite and provided a little DC even without Bacillus. Bentonite, Sand, saw-dust and bagasse showed inert behavior with brinjal plants regarding resistance induction.

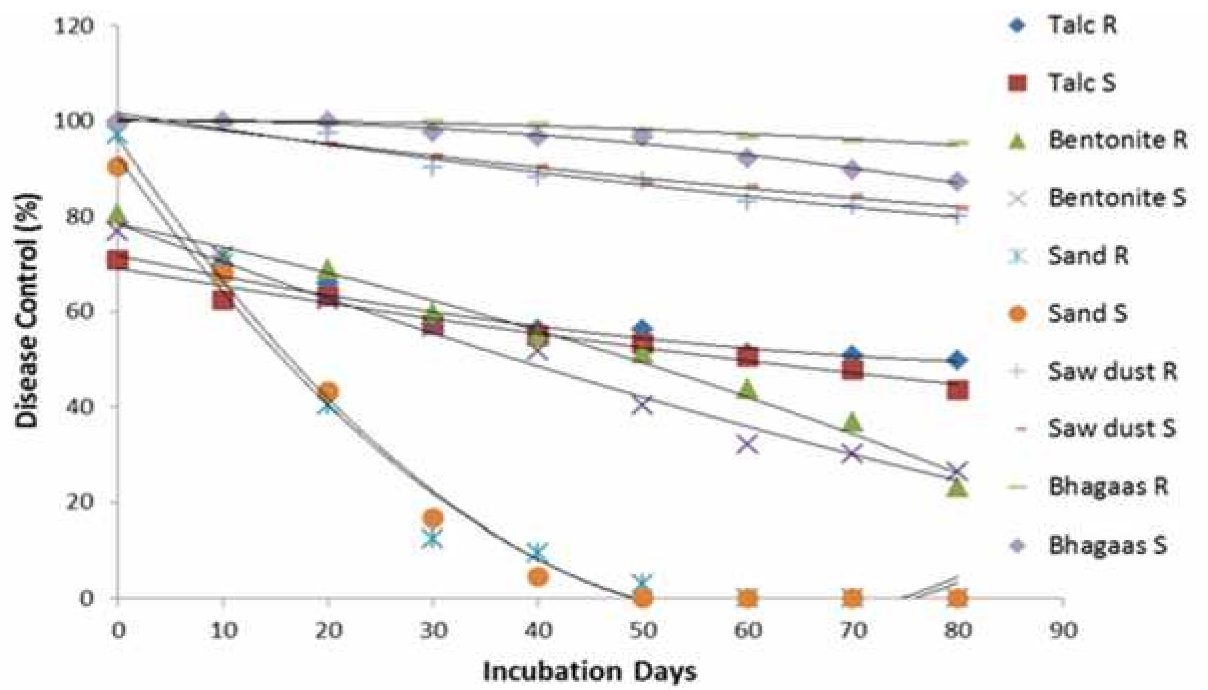


Figure 2.Percentage disease control on brinjal cultivars after application of inducer treatments. Percentage disease control was calculated by putting DS values of pathogen control treatments of (pathogen only) Dilnasheen and Nirala as 16.06 and 3.00, respectively. Data were statistically analyzed through Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT); and LSD value was calculated as 8.62. Polynomial lines were drawn showing trend of each treatment during the time course study.

In comparison of the five carrier materials, bagasse showed the least slope in seven characters out of eight studied. Saw-dust recorded the maximum and persistent support only with respect to the number of leaf hairs (Figure 3H). Dilnasheen was more influenced with inducer treatment than cultivar Nirala in terms of stem diameter, number of fruits/plant, number of branches/ plant and chlorophyll contents (Figure 3B, D, E and G). Plant height, number of leaves/ plant, average weight of fruits and number of leaf hairs were more elevated in Nirala than Dilnasheen under the inducer activity (Figure 3A, C, F and H). Slope of sand was very steep, showing quick decrease in formulation efficiency. Only in case of stem diameter, Bentonite recorded the least efficiency (Figure 3B). Dilnasheen responded to inducer treatment more quickly than Nirala in terms of all studied characters except number of leaf hairs (Figure 3A-G).

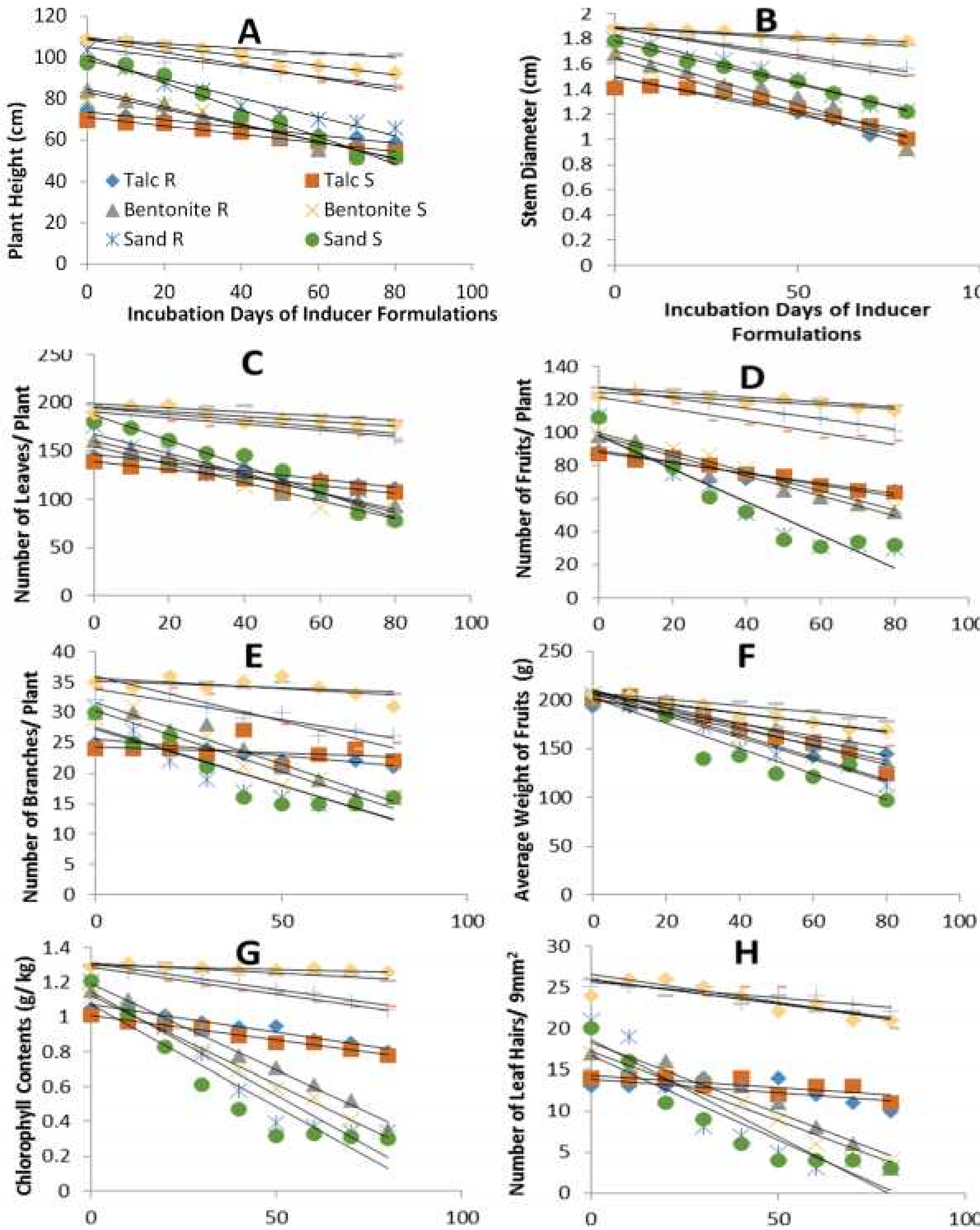


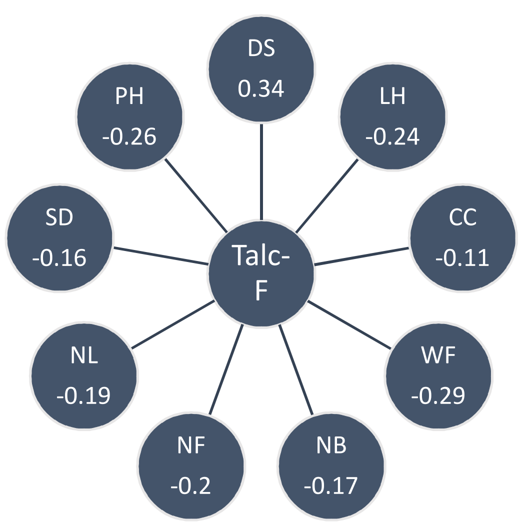
Figure 3. Effects of inducer formulations (prepared in different carrier materials) on height of brinjal plants (A), stem diameter (B), number of leaves/plant (C), number of fruits/plant (D), number of braches/plant (E), average weight of fruits (F), chlorophyll contents (G) and number of leaf hairs (H). Agro-economic features have been mentioned on Y-axis, while incubation days of inducer formulations have been mentioned on X-axis. Data were analyzed and trends were developed using DSAASTAT (Onofri, Italy). Statistical analyses were carried out through Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT); and LSD values were calculated as 10.24, 0.19, 18.64, 11.19, 3.29, 21.24, 0.12, and 2.13, for sections a, b, c, d, e, f, g, and h of this figure, respectively.

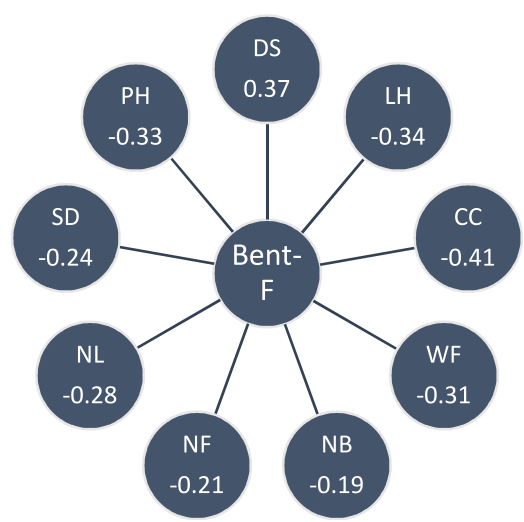
All the tested formulations controlled the disease up to different extents with varied levels of correlations with agroeconomic traits. DS recorded direct correlation with all the formulations; however it showed the maximum correlation with bagasse based formulation. Most of the agroeconomic traits were found in the strongest associations with bagasse formulations except ‘number of leaves’. Number of leaves was the only agroeconomic trait, which had the strongest correlation with saw-dust. Number of fruits was another agroeconomic trait having correlations with bagasse and saw-dust with a very minute difference. Bagasse formulation recorded second highest values of its correlations with agroeconomic traits; while the least values of PCC were calculated in case of Sand based formulation. PCC value of Talc and Bentonite formulations were stronger than correlations of Sand but weaker than of saw-dust. Moreover, the PCC correlations of Bentonite were slightly stronger than of Talc, proving the behavior of both formulations very close to each other in supporting *B. subtilis* (Figure 3).

It was also noticeable that the higher disease severity drastically reduced the number of fruits per plant (-0.62), but it caused only a little reduction in weight of fruits (-0.11). Plant height had the highest negative correlation (-0.71) with DS, whereas chlorophyll contents had slightly weaker negative correlation (-0.67) with DS than of plant height. The third strongest inverse relation was recorded between number of fruits and DS with coefficient value of (-0.62). Leaf hair density was also recorded to be reduced with PCC value of -0.54 as DS was elevated. Stem diameter and number of branches were also in inverse relation with DS raising decimal coefficient values of -0.34 and -0.22, respectively (Figure 5).

Average of grades obtained by individual carrier materials in each agro-economic aspect, depicted bagasse as the best carrier material because it maximum supported *Bacillus* inoculum for boosting 7 out of 8 quality parameters of brinjal. The only parameter to which bagasse stood second in support was “number of leaf hairs” proving less plant resistance against insect pests. Leaf hairs were insignificantly most dense in case of saw-dust which was frequently proved second in supporting potential to *B. subtilis* (Figure 7). Number of fruits was 66.1% more than any other treatment when brinjal were treated with bagasse based inoculum. Moreover, efficiency of bagasse was significantly higher in terms of plant height and number of branches. Whereas, stem diameter, weight of fruits, chlorophyll contents, number of leaves and number of fruits per plant were recorded insignificantly greater with bagasse in comparison to saw-dust treatment (Figure 7). Remaining three carrier materials were inappropriate because of exhibiting least efficiency in one or more agro-economical parameters and none of those three provided the best response in any investigated parameter. Talc treatments provided the least plant height and stem diameters; while, Bentonite treatments were the poorest with reference to number of leaves and fruits per plant. Sand got minimum grades during the measurement of number of branches, weight of fruits, chlorophyll contents and density of leaf hairs (Figure 7).

Total sum of all grades in Figure 7 constructed an overall efficiency index of carrier materials which has been demonstrated in Figure 8. It proved bagasse as significantly the best carrier for *Bacillus* closely followed by saw-dust (Figure 8). Talc could be denoted as third among the list of carrier materials with insignificant superior performance as Sand. While, the least carrying potential for *B. subtilis* was of Bentonite with non-significance difference of control; which had significant difference with Talc but non-significant difference with Sand (Figure 8).





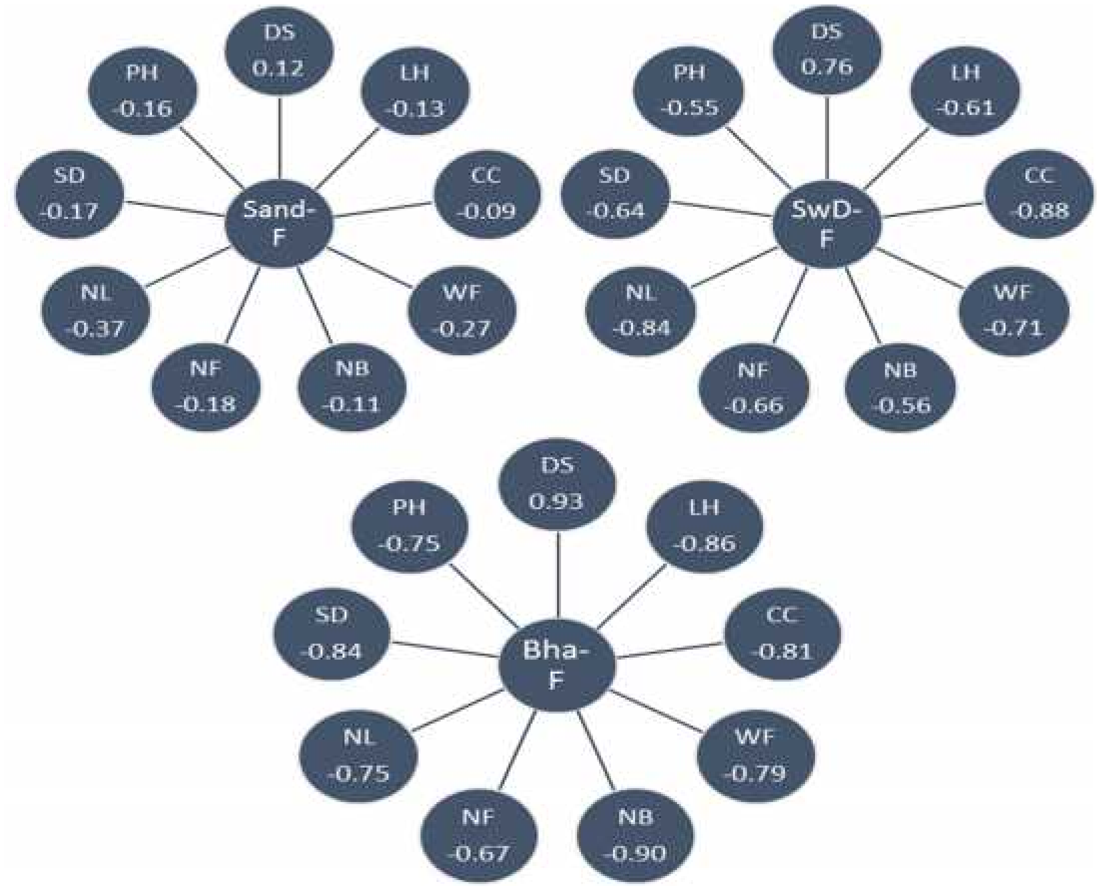


Figure 4. Correlation of microbial activity in different formulations and induction of agroeconomic traits was performed through Pearson's Correlation Coefficient (PCC) analysis. Formulations were prepared in different carrier materials shown by the short terms (Talc-F) Talc, (Bent-F) Bentonite, (Sand-F) Sand, (SwD-F) saw-dust and (Bha-F) bagasse. Agroeconomic traits against which the correlation was checked were denoted as (DS) disease severity, (PH) plant height (SD) stem diameter, (NL) number of leaves, (NF) number of fruits, (NB) number of branches, (WF) weight of fruits, (CC) chlorophyll contents and (LH) leaf hairs.

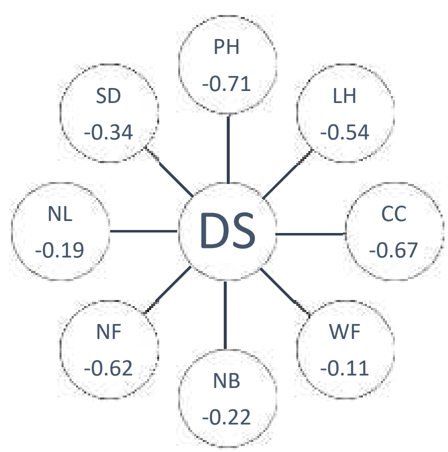


Figure 5. Effect of disease severity (DS) calculated through Pearson's Correlation Coefficient (PCC) on agroeconomic traits of brinjal crop. Negative values show the inverse relation between two parameters; and higher negative values represent stronger inverse relations of agroeconomic traits with disease.

B. subtilis retained its resistance induction potential and growth promotion abilities more proficiently in bagasse (Figure6); while saw-dust proved to be the second most supporting carrier material. The steepest slope of sand revealed that it was the least supporting carrier material for *B. subtilis*.

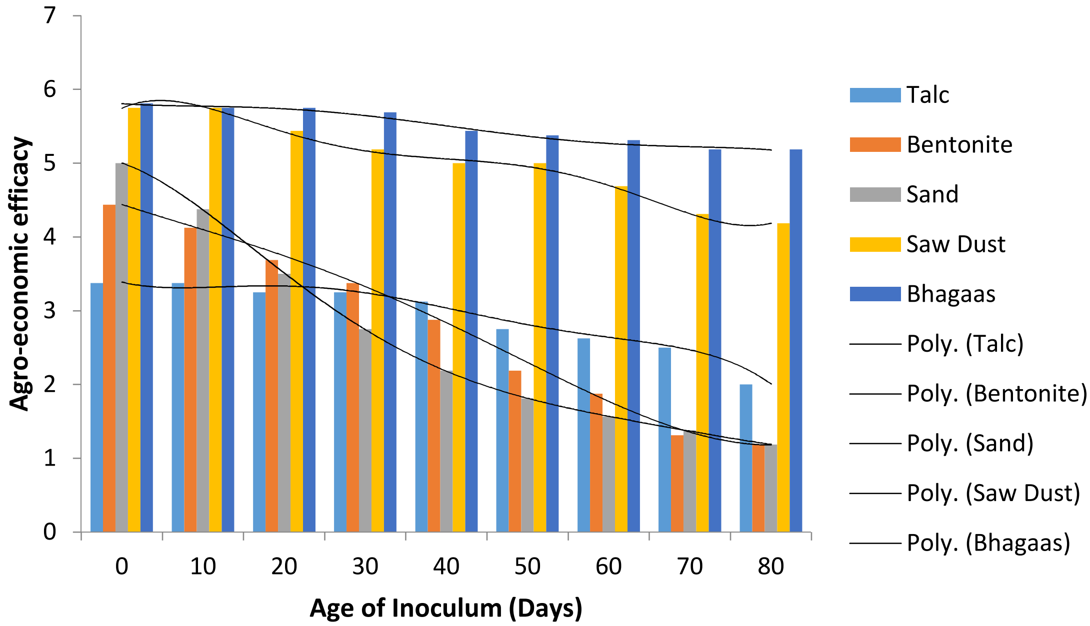


Figure 6. Overall efficiency of carrier materials with respect to incubation/storage period of inoculum at room temperature. Average efficiency of each material was calculated through scores given in 'Table 1' and plotted along Y-axis, while different incubation periods were plotted along X-axis to produce a time course view of reduction in inducer's features. Data were statistically analysed through Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) and LSD value was calculated 2.69.

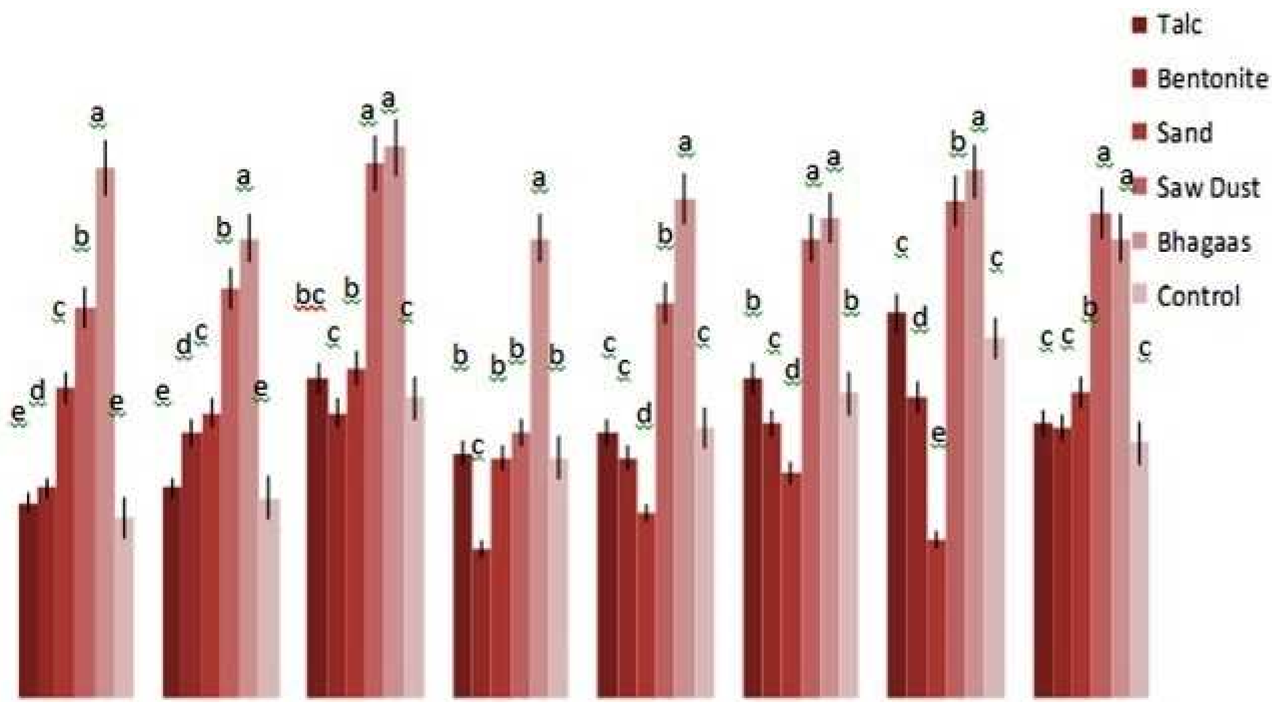


Figure 7. Individual supporting potential of carrier materials to *B. subtilis* for enhancing brinjal agro-economical parameters.

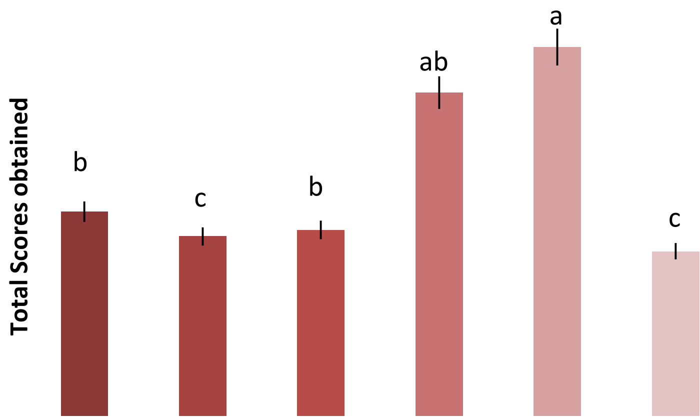


Figure 8.Overall grading of the carrier materials towards resistance induction potential of *B. subtilis* in brinjal against *M. incognita*.

DISCUSSION

The best suitable carrier material can support all the biological features of the preserved species. Alterations of carrier material in a biocontrol formulation may affect the efficacy and viability of its biological agent. Current study exactly explains the varying disease control behavior of *B. subtilis* due to consumption of different carrier materials in its formulation. All carrier materials have their specific chemical and physical properties, which may result into deviations in nematode characteristics (Espinel-Ingroff *et al.,* 2004).Hence, it can be easily demonstrated that how the inoculum of single bacterial species produced different disease control efficiencies when its inoculum carried by different carrier materials was applied to the brinjal plants.

Variations in physical conditions of preservation have a major role in defining conservation of the features of preserved inducer species (Tsironi *et al.,* 2009).Thus, selection of carrier material and incubation conditions for inducer formulations is based upon conservation of resistance induction characteristics for longer periods. Single carrier material may have different preservation efficacy towards different inducers. Similarly, different carrier materials may have different viable incubation periods for a single inducer species (Ranasingh *et al.,* 2006).Bagasse and saw-dust have been proved as efficient carrier materials, by keeping in view all the recorded facts of this study. Storage of inducer formulations at low temperatures is an expensive and difficult task. Therefore, current investigation has evaluated carrier materials at room temperatures. Thus, it has screened out the best carrier material with the least storage expenditures.

Moreover, PCC values show the strength of association between two dependent phenomena. Therefore, higher PCC values of bagasse formulation proved a strong relation between DS and inoculum of *B. subtilis*. Correlation analysis revealed that disease control (DC) through bagasse and saw-dust formulations is highly dependent upon incubation days of biocontrol agent.

Plant height and chlorophyll contents were the most sensitive parameters of this study because they were in a very strong inverse relation with DS. Moreover, it can also be estimated that during the disease, rate of photosynthesis is more affected by the reduction in chlorophyll contents and less by the photosynthetic area, because inverse relation of chlorophyll contents was much higher than inverse relation of number of leaves. Increase in DS will decrease the plant height, and will show little reduction in number of branches, giving the plant a bushy stature. Hence, PCC analysis of agroeconomic traits statistically proves the transformation of brinjal plants into bushy stature due to attack of nematodes. Moreover, a weaker correlation between stem diameter and DS proved the transformation of brinjal plants into a bushy stature. Density of leaf hairs was recorded to be reduced with increased DS, proving weaker plant resistance against herbivory. It supports the phenomenon that fungal diseases may support insect attack on a plant by reducing its resistance against arthropods (Shafique *et al.,* 2014).

Sometimes, carrier materials themselves have DC potential (and become synergistic to inducer) due to their resistance induction abilities (Sharma *et al.*, 2011). It might be the reason behind DC recorded by CC treatment of Talc. It was noticeable that inducer improved plant vigor and agro-economictraits. This finding of present investigation falls in agreement with Islam *et al.,* (2011) Number of leaf hairs was reduced by the application of bagasse formulation. It may decrease plant resistance against insect pests (Rahman, 2002). This is an important aspect in terms of insect transmitted diseases and herbivory. Bagasse formulations cannot be recommended in areas where viral or insect transmitted diseases occur frequently. For such areas, saw-dust based formulations could be the best biocontrol treatments because it did not affect number of leaf hairs, and maintained the plant resistance against herbivory.

Acknowledgements: I am really thankful to the Higher Education Commission (HEC) for funding throughout the research and also I am also grateful to Institute of Agricultural Sciences (IAGS) University of the Punjab, Pakistan for providing me the lab facilities and place to perform this research work.

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