Storability of *Metarhizium anisopliae* with Pesticides

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ABSTRACT

The present investigation's on "Storability of *Metarhizium anisopliae*" were carried out at Biocontrol Laboratory, Agricultural Entomology Section, College of Agriculture, Dhule during Jan 2018 to Dec 2018. The pure culture of *Metarhizium anisopliae* was maintained at Agricultural Entomology Section, College of Agriculture, Dhule on PDA slants . The storability of *M. anisopliae* was studied at 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180 days after storage kept at ambient and freeze temperature. The CFU count was taken by serial dilution technique at 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 dilution factor after 3 days of inoculation. At 15 day's interval 1 gm sample was taken from both room and freeze temperature samples and the viability of the product was studied by serial dilution technique. The results clearly indicated that the viability of the product kept in ambient temperature was 120 days while the viability of the product kept in freeze temperature was 165 days. Initial one month the difference in CFU count between the samples kept at room and freeze temperature was less as compared to later stage of storage. *M. anisopliae* can be stored safely up to 4 months at ambient temperature and maximum up to 5.5 months at freeze temperature.

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Key words: *Metarhizium anisopliae*, serial dilution technique, Storability.

INTRODUCTION

Fungi which control the insect pest population are associated with agricultural crops are considered as entomopathogenic fungi. There are several naturally occurring organisms, such as bacteria, viruses and fungi for the control of crop pest, weeds and plant diseases. *Metarhizium anisopliae* (Metschnikoff) Sorokin, initially known under the name *Entomophthora anisopliae*, was first described near Odessa in Ukraine from infected larvae of the wheat cockchafer *Anisopliae austriaca* in 1879, and later on, *Cleonus punctiventis* by Metschnikoff. It was later renamed as *M. anisopliae* by Sorokin in 1883 (Tulloch, 1976). *Metarhizium* causes a disease known as 'green muscardine' in insect hosts because of the green colour of its conidial cells. In 1883, Metschnikoff commenced mass culturing of fungus and carried out the first experiment with two beetle pests. *Metarhizium anisopliae* (Metschnikoff) Sorokin is the second most widely exploited entomopathogenic fungus in biocontrol trials. Species within the genus *Metarhizium* are pathogenic fungi with broad ranges of insect hosts. *M. anisopliae* was found to be a species complex composed of nine species based on multilocus phylogeny (Bischoff *et al.*, 2009). It is known to attack over 200 species of insects belonging to orders Coleoptera, Dermaptera, Homoptera, Lepidoptera and Orthoptera (Sahayaraj and Borgio, 2008). Many entomopathogenic fungi especially *Metarhizium anisopliae* are used biological control agents of insects including gregarious insect pests. But field application of fungi cannot give satisfactory results as pesticides due to many abiotic and biotic factors (Ferron, 1978; Villani *et al.*, 1992; Anderson and Roberts, 1983; Loria *et al.*, 1983; Alves and Lecuona, 1998).

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MATERIAL AND METHODS

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The study was conducted under laboratory condition at Agricultural Entomology Section, College of Agriculture, Dhule. *M. anisopliae* was inoculated in sterilized potato dextrose broth (PDB) in a saline bottle. After 15 days of inoculation, 11.50 gm of the fully grown mat of *M.*

anisopliae was mixed in 1 kg of talcum powder and 1.15% wettable powder formulation was prepared. 1 kg sample of *M. anisopliae* 1.15% WP was kept at ambient temperature (25^0C to 40^0C) and 1 kg sample of *M. anisopliae* 1.15% WP was kept in refrigerator at 10^0C to 12^oC. After every 15 days interval 1 gm sample from ambient temperature and 1 gm from freeze temperature were used for serial dilution to test the viability of the product. The CFU count was recorded at 3rd day of inoculation on PDA.

Statistical analysis was carried out by analyzing the available data in Factorial Completely Randomised Design (FCRD). The data was subjected to square root transformation prior to analysis.

RESULT AND DISCUSSION

Storability of *M. anisopliae* after 3 days of Inoculation.

After 15 days of storage

The data revealed that the mean CFU count (60.92) was significantly more in sample kept at freeze temperature than the samples kept at ambient temperature (59.13).

The maximum (114.13) mean CFU count was observed in dilution factor 10^4 while the minimum (21.50) mean CFU count was observed in dilution factor 10^9. The dilution factor 10^8 shown (27.13) mean CFU count.

The interaction between temperature factor and dilution factor was non significant at 15 days after storage.

After 30 days of storage

The maximum (58.83) mean CFU count was observed in the samples kept at freeze temperature and was significantly superior over the sample kept at ambient temperature (55.83).

The maximum (112.88) mean CFU count was observed in a dilution factor 10^4 and minimum (18.63) mean CFU count was observed in 10^9. The dilution factor 10^8 shown (26.25) mean CFU count.

The maximum (114.00) mean CFU count was observed in a interaction between the sample kept in freeze temperature and dilution factor 10^4 and was significantly superior over rest of the interactions.

After 45 days of storage

The maximum (57.08) mean CFU count was observed in the samples kept at freeze temperature and was significantly superior over the sample kept at ambient temperature (52.67).

The maximum (111.00) mean CFU count was observed in a dilution factor 10^4 and minimum (15.25) mean CFU count was observed in 10^9. The dilution factor 10^8 shown (22.13) mean CFU count.

The maximum (112.50) mean CFU count was observed interaction between the sample kept in freeze temperature and dilution factor 10^4 and was significantly superior over rest of the interactions.

After 60 days of storage

The maximum (52.50) mean CFU count was observed in the samples kept at freeze temperature and was significantly superior over the sample kept at ambient temperature (46.42).

The maximum (97.25) mean CFU count was observed in a dilution factor 10^4 and minimum (12.88) mean CFU count was observed in 10^9. The dilution factor 10^8 shown (18.25) mean CFU count.

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The maximum (104.00) mean CFU count was observed in interaction between the sample kept at freeze temperature and dilution factor 10^4 and was significantly superior over rest of the interactions.

After 75 days of storage

The maximum (50.58) mean CFU count was observed in the samples kept at freeze temperature and was significantly superior over the sample kept at ambient temperature (41.71). The maximum (91.63) mean CFU count was observed in a dilution factor 10^4 and minimum (12.00) mean CFU count was observed in 10^9. The dilution factor 10^8 shown (16.00) mean CFU count.

The maximum (99.25) mean CFU count was observed in interaction between the sample kept at freeze temperature and dilution factor 10^4 and was significantly superior over rest of the interactions.

After 90 days of storage

The maximum (48.63) mean CFU count was observed in the samples kept at freeze temperature and was significantly superior over the sample kept at ambient temperature (37.25)

The maximum (87.13) mean CFU count was observed in a dilution factor 10^4 and minimum (11.13) mean CFU count was observed in 10^9. The dilution factor 10^8 shown (14.38) mean CFU count.

The maximum (94.50) mean CFU count was observed in interaction between the sample kept at freeze temperature and dilution factor 10^4 and was significantly superior over rest of the interactions.

After 105 days of storage

The maximum (43.46) mean CFU count was observed in the samples kept at freeze temperature and was significantly superior over the sample kept in ambient temperature (33.67).

The maximum (78.38) mean CFU count was observed in a dilution factor 10^4 and minimum (9.38) mean CFU count was observed in 10^9. The dilution factor 10^8 shown (12.88) mean CFU count.

The maximum (85.25) mean CFU count was observed in interaction between the sample kept at freeze temperature and dilution factor 10^4 and was significantly superior over rest of the interactions.

After 120 days of storage

The maximum (36.88) mean CFU count was observed in the samples kept at freeze temperature and was significantly superior over the sample kept in ambient temperature (25.92).

The maximum (66.75) mean CFU count was observed in a dilution factor 10^4 and minimum (6.13) mean CFU count was observed in 10^9. The dilution factor 10^8 shown (10.38) mean CFU count.

The maximum (77.00) mean CFU count was observed in interaction between the sample kept at freeze temperature and dilution factor $10^4$and was significantly superior over rest of the interactions.

After 135 days of storage

The mean CFU count (28.38) was observed in the samples kept at freeze temperature and was significantly superior over the sample kept at ambient temperature (6.50).

The maximum (45.25) mean CFU count was observed in a dilution factor 10^4 and minimum (3.00) mean CFU count was observed in 10^9. The dilution factor 10^8 shown (6.00) mean CFU count. The maximum (64.25) mean CFU count was observed in a interaction between

the sample kept at freeze temperature and dilution factor 10^4 and was significantly superior over rest of the interactions.

After 150 days of storage

The maximum (14.00) mean CFU count was observed in the samples kept at freeze temperature and was significantly superior over the sample kept in ambient temperature (1.58).

The maximum (21.63) mean CFU count was observed in a dilution factor 10^4 and minimum (2.25) mean CFU count was observed in 10^9. The dilution factor 10^8 shown (3.25) mean CFU count.

The maximum (33.55) mean CFU count was observed in interaction between the sample kept at freeze temperature and dilution factor 10^4 and was significantly superior over rest of the interactions.

After 165 days of storage

The maximum (8.96) mean CFU count was observed in the samples kept at freeze temperature and was significantly superior over the sample kept in ambient temperature (00.00).

The maximum (10.63) mean CFU count was observed in a dilution factor 10^4 and minimum (0.75) mean CFU count was observed in 10^9. The dilution factor 10^8 shown (1.50) mean CFU count.

The maximum (21.25) mean CFU count was observed in interaction between the sample kept at freeze temperature and dilution factor 10^4 and was significantly superior over rest of the interactions.

After 180 days of storage

The CFU count of freeze temperature was (0.00) which indicates storability of freeze product was up to 165 days.

The overall results on storability of *M. anisopliae* at ambient and freeze temperature clearly indicate that at room temperature upto 120 days i.e 4 months the viability of *M. anisopliae* is good but later on at 135 days after storage it drastically reduced and at 150 days i.e 5 months after preparation of the finished product lost it's viability. While in refrigerator storage it may last upto 165 days also.

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Bell and Hamalle (1974) reported that *B. bassiana, M. anisopliae* and *Spicaria rilevi* retained their pathogenicity to insects after 3 years also. In present investigation the storability of *M. anisopliae* was upto 4 months at ambient temperature.

Muller-Kogler *et al* (1980) reported that storage at 18°C proved the most satisfactory for fungus imperfecti (including *M. anisopliae, B. bassiana* and *N. ri1eyi*) and some of the groups were still alive after a year. Sporulation and growth of subculturing after storage were comparable to those of the original cultures.

Muller-Kogler and Zimmermann (1980) evaluate 12 isolates each of *M. anisopliae, B, bassiana, E. brongniartii* and *Hirsutella spp*, were stored at 4^0C all of the isolates survived for 3 years. The present investigation were carried out at room temperature at 25^oC to 40^oC and freeze temperature at 10 to 12^oC.

Alves *et.al.* (1987) reported that the time for which *M. anisopliae* would be stored increased by up to 33 per cent at room temperature and up to 52 per cent in refrigerator (at 2-3^oC), depending on formulation and formulation kept in the freezer were viable up to 660 days (70 % viability). In present investigation also when the samples kept at room temperature remain viable upto 135 days only while the sample kept in refrigerator the viability of *M. anisopliae* remain upto 165 days which are in confirmation with present findings.

The Findings reported by the above workers are in agreement with the recent study. More or less due to variability in test conditions and formulations.

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CONCLUSION

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From the present investigation on storability studies of *Metarhizium anisopliae* it is concluded that the storage ability of sample of *Metarhizium anisopliae* kept in both room and freeze temperature starts to decline with respect to time. Storage ability of sample of *Metarhizium anisopliae* kept in freeze temperature is found greater than that of sample of *Metarhizium anisopliae* kept in room temperature. Storage ability of sample of *Metarhizium anisopliae* kept in freeze temperature was about 165 days from production of finished product whereas storage ability of sample of *Metarhizium anisopliae* kept in room temperature was 120 days only.

REFERENCES

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Alves, R. T., Prior, C. and Leather, S. R. 1987. Evasion of host defence by in vivo-produced protoplast like cell of the insect mycopathogen *Beauveria bassiana*. J. Biocontrol Sep, **175**(18):5962-5969.

Alves, S.B. and Lecuona, R.E. 1998. Epizootiologia aplicadaao controle microbiano Applied epizootiology microbial control. p. 97–157. In: Alves, S.B., ed. Controlemicrobiano de insetos Microbial control of insects. Fealq, Piracicaba, SP, Brazil.

Anderson, T.E. and Roberts, D.W. 1983. Compatibility of *Beauveria bassiana* isolate with insecticide formulations used in Colorado potato beetle (*Coleotera: Crysomelidae*) Control. *Journal of Economic Entomology*, **76**: 1437–1441.

Bell, J.V. and Hamalle, R.J. 1974. Viability and pathogenicity of entomogenous fungi after prolonged storage on silica gel at −20° C. *Canadian journal of microbiology*, **20**(5), pp.639-642.

Bischoff, J.F., Rehner, S.A. and Humber, R.A. 2009. A multilocus phylogeny of *Metarhizium anisopliae* lineage. *Mycologia*, **101**:512-530.

Ferron, P. 1978. Biological control of insect pests by entomogenous fungi. *Annual Review Entomology*, **23**: 409-442.

Loria, R., Galaini, S. and Roberts, D. W. 1983. Survival of inoculum of the entomopathogenic fungus *Beauveria bassiana*as influenced by fungicides. *Environtal Entomology*, **12**:

Müller-Kögler, E. and Zimmermann, G., 1980. Zur aufbewahrung entomogener pilzkulturen. *Entomophaga*, **25**(3):301-311.

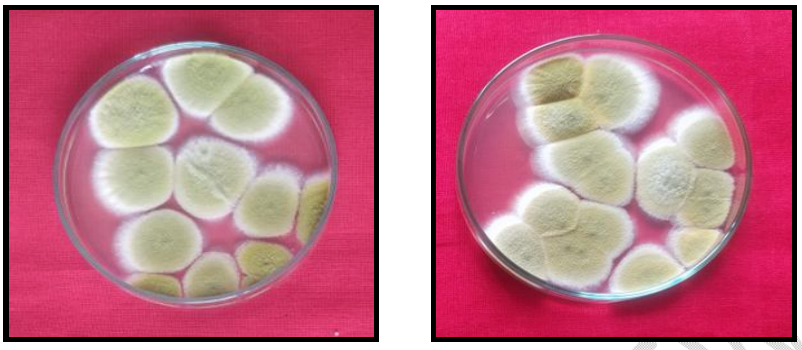
Müller-Kögler, E., Zimmermann, G., and Mortin, P.A. 1980. On the storage of cultures of entomopathogenic fungi. Entomophaga, **25**: 3, 301-311, 2 ref.

Sahayaraj, K. and Borgio, J. F. 2008. Tri-tropic interaction of cotton, red cotton bug and green muscardine fungi under in-vitro condition. *Journal of Biopesticides*, **1**(1): 41-46.

Tulloch, M. 1976. The genus *Metarhizium anisopliae. Transaction of the Britannic Mycology Society*, **66**: 407-411.

Villani, M. G., Krueger, S. R. and Nyrop, J. P. 1992. A case study of the impact of the soil environment on insect/pathogen interactions: Scarabs in turfgrass. In: use of

pathogens in Scarab pest management. (Eds.): T.R. Glare and T.A. Jackson. Intercept, Hampshire, pp. 111-126.



(A) (B)

Plate 1: CFU Count of *M. anisopliae* after 105 days of storage at dilution factor of (10^8) at room (A) and freeze temperature (B).

(A) (B)

Plate 2: CFU Count of *M. anisopliae* after 135 days of storage at dilution factor of (10^8) at room (A) and freeze temperature (B)

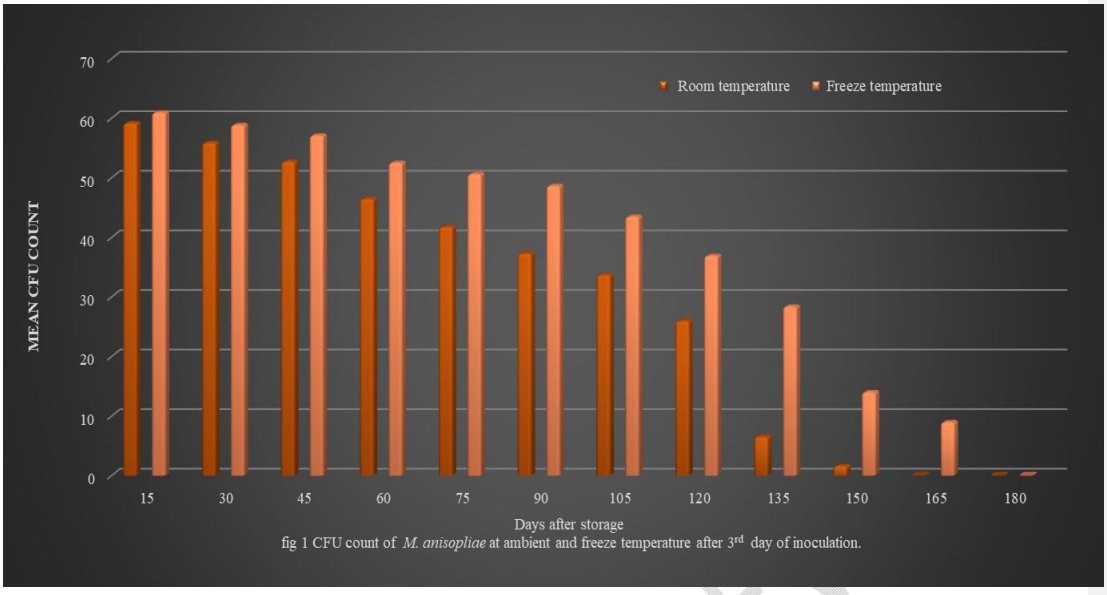


fig 1 CFU count of *M. anisopliae* at ambient and freeze temperature after 3rd day of inoculation.

Table 1: Storability of *Metarhizium anisopliae* at room and freeze temperature after 3 days of inoculation.

Factors	**CFU count of *M. anisopliae* days after finished product preparation**											
Factor A (T) (Temperature)	15 days	30 days	45 days	60 days	75 days	90 days	105 days	120 days	135 days	150 days	165 Days	180 Days
(T_1) Room temperature	59.13 (7.42) *	55.83 (7.12)	52.67 (6.90)	46.42 (6.47)	41.71 (6.11)	37.25 (5.77)	33.67 (5.49)	25.92 (4.76)	6.50 (1.94)	1.58 ()1.12	0.00 (0.71)	0.00 (0.71)
(T_2) Freeze temperature	60.92 (7.52)	58.83 (7.36)	57.08 (7.21)	52.50 (6.89)	50.58 (6.77)	48.63 (6.62)	43.46 (6.24)	36.88 (5.72)	28.38 (5.06)	14.00 (3.60)	8.96 (2.87)	0.00 (0.71)
S. E. $^{+}_{-}$	**0.027**	**0.026**	**0.026**	**0.025**	**0.030**	**0.032**	**0.028**	**0.036**	**0.034**	**0.038**	**0.025**	**0.00**
C. D. at 5%	**0.078**	**0.075**	**0.074**	**0.074**	**0.087**	**0.094**	**0.081**	**0.103**	**0.097**	**0.109**	**0.072**	**NS**
Factor B (D) Dilution factor												
(D_1) 10^{-4}	114.13 (10.71)	112.88 (10.65)	111.00 (10.56)	97.25 (9.88)	91.63 (9.59)	87.13 (9.35)	78.38 (8.87)	66.75 (8.18)	45.25 (6.61)	21.63 (4.50)	10.63 (2.68)	0.00 (0.71)
(D_2) 10^{-5}	88.63 (9.44)	86.75 (9.34)	86.63 (9.33)	80.00 (8.97)	75.75 (8.72)	70.00 (8.36)	63.88 (7.99)	54.88 (7.41)	26.50 (5.01)	9.38 (2.54)	6.75 (2.22)	0.00 (0.71)
(D_3) 10^{-6}	64.63 (8.07)	61.13 (7.85)	57.25 (7.60)	54.13 (7.39)	48.88 (7.02)	44.88 (6.71)	39.38 (6.28)	30.38 (5.53)	13.38 (2.96)	6.00 (2.12)	4.25 (1.85)	0.00 (0.71)
(D_4) 10^{-7}	44.13 (6.68)	38.00 (6.20)	37.00 (6.12)	34.25 (5.89)	32.63 (5.75)	30.13 (5.53)	27.50 (5.29)	19.88 (4.51)	10.50 (2.67)	4.25 (1.85)	3.00 (1.63)	0.00 (0.71)
(D_5) 10^{-8}	27.13 (5.26)	26.25 (5.17)	22.13 (4.75)	18.25 (4.33)	16.00 (4.05)	14.38 (3.85)	12.88 (3.65)	10.38 (3.27)	6.00 (2.12)	3.25 (1.67)	1.50 (1.28)	0.00 (0.71)
(D_6) 10^{-9}	21.50 (4.69)	18.63 (4.67)	15.25 (3.96)	12.88 (3.64)	12.00 (3.51)	11.13 (3.38)	9.38 (3.12)	6.13 (5.53)	3.00 (1.63)	2.25 (1.47)	0.75 (1.06)	0.00 (0.71)
S. E. $^{+}_{-}$	**0.016**	**0.016**	**0.015**	**0.015**	**0.018**	**0.020**	**0.017**	**0.022**	**0.020**	**0.023**	**0.015**	**0.00**
C. D. at 5%	**0.048**	**0.046**	**0.045**	**0.045**	**0.052**	**0.057**	**0.050**	**0.063**	**0.059**	**0.067**	**0.044**	**NS**
Interaction												
T_1D_1	113.25 (10.67)	111.75 (10.59)	109.50 (10.49)	90.50 (9.54)	84.00 (9.19)	79.75 (8.95)	71.50 (8.49)	56.50 (7.55)	26.25 (5.17)	9.50 (3.16)	0.00 (0.71)	0.00 (0.71)
T_1D_2	86.50 (9.33)	83.25 (9.15)	82.00 (9.08)	74.50 (8.66)	67.50 (8.25)	57.25 (7.60)	53.00 (7.31)	44.50 (6.71)	12.75 (3.63)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)
T_1D_3	62.50	57.50	54.00	52.50	44.50	37.25	31.50	25.25	0.00	0.00	0.00	0.00

	(7.94)	(7.62)	(7.38)	(7.28)	(6.70)	(6.14)	(5.65)	(5.07)	(0.71)	(0.71)	(0.71)	(0.71)
T_1D_4	44.00 (6.67)	37.25 (6.14)	35.75 (6.02)	32.50 (5.74)	31.25 (5.63)	28.50 (5.38)	27.25 (5.27)	17.50 (4.24)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)
T_1D_5	27.00 (5.24)	26.00 (5.15)	21.00 (4.63)	17.75 (4.27)	13.75 (3.77)	12.50 (3.60)	11.25 (3.43)	7.75 (2.87)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)
T_1D_6	21.50 (4.69)	18.50 (4.36)	13.75 (3.77)	10.75 (3.35)	9.25 (3.12)	8.25 (2.95)	7.50 (2.82)	4.00 (2.11)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)
T_2D_1	115.00 (10.75)	114.0 (10.70)	112.50 (10.63)	104.00 (10.22)	99.25 (9.99)	94.50 (9.75)	85.25 (9.26)	77.00 (8.80)	64.25 (8.04)	33.55 (5.85)	21.25 (4.66)	0.00 (0.71)
T_2D_2	90.75 (9.55)	90.25 (9.53)	91.25 (9.58)	85.50 (9.27)	84.00 (9.19)	82.75 (9.12)	74.75 (8.66)	65.25 (8.11)	40.25 (6.38)	18.75 (4.38)	13.50 (3.74)	0.00 (0.71)
T_2D_3	66.75 (8.20)	64.75 (8.08)	60.50 (7.81)	55.75 (7.50)	53.25 (7.33)	52.50 (7.28)	47.25 (6.91)	35.50 (6.00)	26.75 (5.22)	12.00 (3.53)	8.50 (3.00)	0.00 (0.71)
T_2D_4	44.25 (6.69)	38.75 (6.26)	38.25 (6.22)	36.00 (6.04)	34.00 (5.87)	31.75 (5.68)	27.75 (5.29)	22.25 (4.77)	21.00 (4.64)	8.50 (3.00)	6.00 (2.55)	0.00 (0.71)
T_2D_5	27.25 (5.27)	26.50 (5.19)	23.25 (4.87)	18.75 (4.39)	18.25 (4.33)	16.25 (4.09)	14.50 (3.87)	13.00 (3.67)	12.00 (3.53)	6.50 (2.64)	3.00 (1.86)	0.00 (0.71)
T_2D_6	21.50 (4.69)	18.75 (4.39)	16.75 (4.15)	15.00 (3.94)	14.75 (3.90)	14.00 (3.81)	11.25 (3.43)	8.25 (2.95)	6.00 (2.55)	4.50 (2.23)	1.50 (1.40)	0.00 (0.71)
S. E. $^{+}_{-}$	**0.23**	**0.064**	**0.06**	**0.063**	**0.074**	**0.080**	**0.06**	**0.08**	**0.083**	**0.093**	**0.061**	**0.00**
C. D. at 5%	**N.S.**	**0.18**	**NS**	**0.181**	**0.214**	**0.23**	**0.20**	**0.25**	**0.0239**	**0.269**	**0.176**	**NS**

Note*: Figures in parenthesis are $\sqrt{x+0.5}$ transformed value