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EFFECT OF INVIGORATION TREATMENT TO THE DETERIORATING MUSTARD SEED DUE TO *ASPERGILLUS FLAVUS* ON THE GROWTH AND BIOCHEMICAL CONSTITUENTS OF THE SEEDLINGS

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On storage of mustard Pusa 256 seeds, having 7.86% moisture content and possessing 98% germinability, with *Aspergillus flavus* at 60, 70 and 80% RH at 30 ± 1°C for 20 days, gradual significant decrease in germination, length and dry weight of seedlings, total chlorophyll, total soluble sugar and total free amino acid content in them besides nitrate reductase and urease activities were recorded. After treatment of such deteriorated seeds with 2% solution of $CuSO_4$, $FeSO_4$, KH_2PO_4, $MgSO_4$, $(NH_4)_6Mo_7O_{24}$ and $(NH_2)_2CO$, and 50 ppm solution of IAA separately, significant enhancement in the above aspects of the seedlings was recorded. The germination was observed highest (88%) of the worst deteriorated seeds (76% germination) stored at 80% RH due to $(NH_4)_6Mo_7O_{24}$ and $(NH_2)_2CO$, length due to $(NH_2)_2CO$ (28.25 cm against 19.76 cm of control), dry weight due to $(NH_4)_6Mo_7O_{24}$ (17.68 mg against 8.76 mg of control), total chlorophyll (0.73 mg against 0.65 mg of control) and total soluble sugar (1.71 % conc. against 1.06% of control) due to $MgSO_4$, and total free amino acid (2.36% conc. against 0.40% of control) due to $(NH_2)_2CO$ and activity of nitrate reductase (0.66% conc. of KNO_2 against 0.42% conc. of control). Urease activity was recorded highest due to $(NH_2)_2CO$.

Keywords: *Aspergillus flavus*; Biochemicals; Germination; Growth; Invigoration; Mustard Pusa 256 seed; RH.

Introduction

Seeds have been reported to be deteriorated disturbing the physiology and biochemistry of germination and growth due to the storage fungi[1-8]. Based on the idea of ameliorating effect of invigoration treatment to the seeds producing weak and physiologically subnormal seedling, with some salts and organic compounds[9-11], in the present paper, mustard seeds deteriorating due to *Aspergillus flavus* stored at varying RH (%), were treated with the solution of inorganic salts and organic compounds to observe their effect on germination of the seeds and growth of the seedlings produced by them, total chlorophyll (TC), total soluble sugars (TSS) and total free amino acid (TFAA) content and nitrate reductase (NR) and Urease (UR) activities in them.

Material and Methods

Artificial deterioration of seed-Mustard (*Brassica campestris* L) Pusa 256 seeds were procured from Rajendra Agricultural University, Pusa, Bihar state and storage fungi were isolated adopting blotter technique. Of so many storage fungi isolated from the seeds, *Aspergillus flavus* Link ex Fries only, based on its highest frequency (77%) was selected to observe its effect on the aspects mentioned earlier. *A. flavus* was grown on Czapek Dox Agar slants and seeds were infested with the spore suspension in 5% Tween 20 having 1 x 10^6 spores / ml suspension. A lot of 100 g surface sterilized seeds having 7.8% moisture and possessing 98% germinability was infested with 1 ml suspension and thoroughly agitated in sterilized dry conical flasks, maintaining control using 5% Tween 20 only. The seedlots were stored over 60, 70 and 80% RH maintained with the help of glycerol solution in sealed desiccators[12] at 30 ± 1°C for a period of 20 days to afflict different degrees of artificial deterioration.

Preparation of solution for invigoration treatment- Copper sulphate ($CuSO_4.5H_2O$), Ferrous sulphate ($FeSO_4.7H_2O$), Magnesium sulphate ($MgSO_4.7H_2O$) and Ammonium molybdate {$(NH_4)_6Mo_7O_{24}.4H_2O$} were dried at 100°C for

6 hr and cooled over fused calcium chloride for the time to their constant weight in sealed desiccators to remove the water of crystallization. 2% solution of the dried salts as above and Potassium dihydrogen phosphate (KH_2PO_4) and Urea $(NH_2)_2CO$ was prepared in distilled water. Indole acetic acid (IAA) was also prepared in 50 ppm concentration. The seedlots artificially deteriorating due to the said fungus were surface sterilized with 0.1% mercuric chloride for 1 min. and washed four times with autoclaved tap water and finally two times with autoclaved distilled water. The adherent water was removed by pressing lightly with folds of dry sterilized blotting sheets and set for soaking in the solutions separately. 25 g of seedlot was soaked in 50 ml of the solution taking in beaker of 100 ml capacity at room temperature for 12 hr.

Observation of germination and culture of the seedlings - The seeds treated with the invigorating chemicals, were sown in autoclaved garden soil at 15 psi for 20 min on two consecutive days and taken in earthen pots of 20 cm top diameter, 20 cm depth and 15 cm base diameter. Twenty seeds per pot were sown 0.5 cm deep in previously watered soil nearly at equal distance. Ten pots were set for the seeds treated with one chemical. The seeded soil was covered with nearly 0.5 cm thick absorbant cotton wool. The soil was lightly watered every alternate day and the seeds were permitted to germinate keeping the pots in the open garden in the month of October 2007. The account of germination (%) was maintained (Table 1) based on total 200 seeds (20 seeds each pot x 10 pots).

Estimation of growth of the seedlings-After germination the seedlings were permitted to grow for 15 days. Total length and dry weight of the seedlings were considered as the criteria of growth. For the former, five seedlings were taken out from each pot randomly with the help of Khurpi without damaging the root and rootlets. Total length of the seedlings was measured in cm scale and the mean of ten replicates (each replicate consisting of the length of five seedlings) was recorded (Table 1). For determining the dry weight of the seedlings, these were taken out in the manner described for determining the length, and washed cautiously to avoid loss of the fine rootlets, and adherent water was removed by drying with blotting sheets. Seedlings were dried in an incubator at 80°C for 24 hr and cooled over fused calcium chloride in sealed desiccators to their constant weight. The mean of ten replicates (one replicate of the dry weight of five seedlings) was recorded (Table 1).

Estimation of TC, TSS and TFAA in the seedlings - TC of the seedlings was estimated[13] by cutting the first leaves at 2 P.M. and extracting with 80% acetone and recorded (Table 2). TSS[14] and TFAA[15] were determined by extracting the seedlings in warm 80% ethanol and recorded in Table 2.

Assaying NR and UR activities- NR[16] and UR[17] were assayed watering the seedlings with 2% potassium nitrate and urea solution respectively two days ahead of the day of assay. NR was recorded (Table 3) as reduction of 1.0% KNO_3 to KNO_2 per 30 min. of reaction at 30°C in term of concentration of the latter. The activity of UR was recorded as change in O.D./min. due to oxidation of $NADPH_2$ to NADP (Table 4-6).

Results and Discussion

The effect of *A. flavus* on storage of seeds at increasing RH (%) indicates gradual decrease in germination of the seed, length and dry weight of the seedlings (Table 1) and TC, TSS and TFAA content (Table 2) therein besides NR (Table 3) and UR (Table 4, 5, 6) activities. Significant ($P = 0.001$) difference exists between the treatment and the control. The germination (%) was recorded (Table 1) enhanced due to the treatment to the deteriorated seeds with solutions. Even the worst deteriorated seedlot (81% germination) resulted in better germination (%) due to IAA (91%) followed by $(NH_4)_6Mo_7O_{24}$ and $(NH_2)_2CO$ (88%). Similarly the difference exist ($P = 0.001$) between the control and the treatment with respect to the length and dry weight (Table 1) of the seedlings. The two parameters of growth were statistically more than those seedlings raised from the untreated control. Maximum length of the seedling (< 28 cm) raised from the worst deteriorated seed exhibiting the minimum length (19.76 cm) was observed due to KH_2PO_4, $MgSO_4$ and $(NH_4)_6Mo_7O_{24}$. $MgSO_4$, $(NH_4)_6Mo_7O_{24}$ and $(NH_2)CO$ produced maximum dry weight (< 17 mg) in comparison to 8.76 mg of the control. TC, TSS and TFAA were also more due to treatment to the deteriorated seeds. $MgSO_4$, $FeSO_4$, $CuSO_4$, IAA and $(NH_2)_2CO$ produced maximum TC (Table 2). $MgSO_4$, KH_2PO_4 and $(NH_4)_6Mo_7O_{24}$ produced maximum TSS and $(NH_2)_2CO$, $(NH_4)_6Mo_7O_{24}$ and $MgSO_4$ resulted in producing maximum TFAA (Table 2). The activity of NR was highest due to $(NH_2)_2CO$ and IAA (Table 3). The activity of UR was also higher due to treatment to the deteriorated seeds than the control, and highest activity was observed due to $(NH_2)_2CO$, $FeSO_4$ and $(NH_4)_6Mo_7O_{24}$ (Table 4-6).

As regards the mycodeterioration of seeds in humid storage, its various aspects have earlier been discussed[6,18-20] and interpreted in terms of profuse growth of the fungus on high moisture seed secreting more toxic principles. The result is the disruption of the normal physiology of germination and growth thereafter. The

Table 1. Effect of invigoration treatment to the mustard seeds stored with *A. flavus* at varying RH on their per cent germination (based on 20 seeds in 10 replicates, figures rounded to their whole number) total length (in cm) and dry weight (in mg) of the seedlings (mean of 10 replicates each of 5 seedlings) raised from them.

Invigorating chemicals	Germination RH (%) levels			Length of seedlings RH (%) levels			Dry Weight of seedlings RH (%) levels		
	60	70	80	60	70	80	60	70	80
$CuSO_4$	96	94	86	23.75	21.34	19.25	13.86	12.18	9.12
$FeSO_4$	96	94	87	29.05	28.85	27.10	17.01	15.33	13.46
IAA	98	96	91	29.75	28.05	27.25	16.32	15.82	13.72
KH_2PO_4	97	95	87	28.62	28.20	28.28	18.92	15.99	16.65
$MgSO_4$	96	94	87	29.80	28.15	28.18	20.22	18.82	17.54
$(NH_4)_6Mo_7O_{24}$	97	94	88	28.30	28.30	27.30	21.67	19.57	17.68
$(NH_2)_2CO$	97	95	88	29.30	29.10	28.25	21.86	19.63	17.35
Control	92	88	76	23.82	22.05	19.76	14.42	12.02	8.76

CD (P = 0.001) = 2.26 for chemicals CD (P = 0.001) = 0.215 for Chemicals CD (P = 0.001) = 0.22 for Chemicals
CD (P = 0.001) = 3.73 for RH CD (P = 0.001) = 0.132 for RH CD (P = 0.001) = 0.133 for RH

Table 2. Effect of invigoration treatment to the mustard seeds stored with *A. flavus* at varying RH on total chlorophyll (mg/g fresh leaf), total soluble sugar (% concentration) and total free amino acid (% concentration) in the seedlings raised from them.

Invigorating chemicals	Total Chlorophyll RH (%) levels			Total soluble sugar RH (%) levels			Total Free amino acid RH (%) levels		
	60	70	80	60	70	80	60	70	80
$CuSO_4$	1.92	1.38	0.70	1.87	1.65	1.24	0.82	0.65	0.48
$FeSO_4$	1.90	1.36	0.72	1.96	1.68	1.18	1.68	1.39	1.03
IAA	1.96	1.42	0.70	1.64	1.38	1.09	1.87	1.72	1.49
KH_2PO_4	1.78	1.48	0.66	2.48	2.18	1.65	2.16	1.87	1.52
$MgSO_4$	1.74	1.39	0.73	2.51	2.25	1.71	2.38	2.09	1.86
$(NH_4)_6Mo_7O_{24}$	1.73	1.41	0.67	2.38	2.17	1.58	2.59	2.41	2.12
$(NH_2)_2CO$	1.75	1.39	0.70	2.18	1.85	1.51	2.71	2.59	2.36
Control	1.62	1.35	0.65	1.51	1.32	1.06	0.72	0.58	0.40

CD (P = 0.005) = 0.080 for Chemicals CD (P = 0.05) = 0912 for Chemicals CD (P = 0.001) = 0.029 for Chemicals
CD (P = 0.001) = 0.132 for RH CD (P = 0.001) = 0.056 for RH CD (P = 0.001) = 0.017 for RH

Table 3. Effect of invigoration treatment to the mustard seeds stored with *A. flavus* at varying RH on the activity of nitrate reductase in the seedlings raised from them (expressed as concentration of KNO_2).

Invigorating chemicals	RH (%)		
	60	70	80
$CuSO_4$	2.42	1.46	0.43
$FeSO_4$	2.56	1.52	0.57
IAA	2.70	1.54	0.65
KH_2PO_4	2.64	1.58	0.59
$MgSO_4$	2.54	1.53	0.53
$(NH_4)_6Mo_7O_{24}$	2.58	1.54	0.62
$(NH_2)_2CO$	2.63	1.60	0.66
Control	2.31	1.36	0.42

CD (P = 0.001) = 0.20 for chemicals
CD (P = 0.001) = 0.13 for RH

Table 4. Activity of urease of the seedlings of mustard raised from the seeds stored with *A. flavus* at 60% RH after their invigoration treatment (expressed as change in O.D. per min. pointing out the oxidation of $NADPH_2$ to NADP).

Sl. No.	Invigorating Chemicals	Time in minute									
		1	2	3	4	5	6	7	8	9	10
1.	$CuSO_4$	1.640	1.641	1.642	1.643	1.644	1.645	1.646	1.647	1.648	1.649
2.	$FeSO_4$	1.691	1.693	1.695	1.697	1.699	1.701	1.703	1.705	1.707	1.709
3.	IAA	1.420	1.422	1.424	1.426	1.428	1.430	1.432	1.434	1.436	1.438
4.	KH_2PO_4	1.650	1.651	1.652	1.653	1.654	1.655	1.656	1.657	1.658	1.659
5.	$MgSO_4$	1.643	1.645	1.647	1.649	1.651	1.653	1.655	1.657	1.659	1.661
6.	$(NH_4)_6Mo_7O_{24}$	1.660	1.662	1.664	1.666	1.668	1.670	1.672	1.674	1.676	1.678
7.	$(NH_2)_2CO$	1.842	1.844	1.846	1.848	1.850	1.852	1.854	1.856	1.858	1.860
8.	Control	1.404	1.406	1.408	1.410	1.412	1.414	1.416	1.418	1.420	1.422

Table 5. Activity of urease of the seedlings of mustard raised from the seeds stored with *A. flavus* at 70% RH after their invigoration treatment (expressed as change in O.D. per min. pointing out the oxidation of $NADPH_2$ to NADP).

Sl. No.	Invigorating Chemicals	Time in minute									
		1	2	3	4	5	6	7	8	9	10
1.	$CuSO_4$	1.621	1.622	1.623	1.624	1.625	1.626	1.627	1.628	1.630	1.631
2.	$FeSO_4$	1.660	1.662	1.664	1.666	1.668	1.670	1.672	1.674	1.676	1.678
3.	IAA	1.390	1.392	1.394	1.396	1.398	1.400	1.402	1.404	1.406	1.408
4.	KH_2PO_4	1.630	1.631	1.632	1.633	1.634	1.635	1.636	1.637	1.638	1.639
5.	$MgSO_4$	1.613	1.615	1.617	1.619	1.621	1.623	1.625	1.627	1.629	1.631
6.	$(NH_4)_6Mo_7O_{24}$	1.620	1.622	1.624	1.626	1.628	1.630	1.632	1.634	1.636	1.638
7.	$(NH_2)_2CO$	1.792	1.794	1.796	1.798	1.800	1.802	1.804	1.806	1.808	1.810
8.	Control	1.356	1.358	1.360	1.362	1.364	1.366	1.368	1.370	1.372	1.374

Table 6. Activity of urease of the seedlings of mustard raised from the seeds stored with *A. flavus* at 80% RH after their invigoration treatment (expressed as change in O.D. per min. pointing out the oxidation of $NADPH_2$ to NADP).

Sl. No.	Invigorating Chemicals	Time in minute									
		1	2	3	4	5	6	7	8	9	10
1.	$CuSO_4$	1.609	1.610	1.611	1.612	1.613	1.614	1.615	1.616	1.617	1.618
2.	$FeSO_4$	1.640	1.642	1.644	1.646	1.648	1.650	1.652	1.654	1.656	1.658
3.	IAA	1.390	1.392	1.394	1.396	1.398	1.400	1.402	1.404	1.406	1.408
4.	KH_2PO_4	1.611	1.613	1.615	1.617	1.619	1.621	1.623	1.625	1.627	1.629
5.	$MgSO_4$	1.602	1.604	1.606	1.608	1.610	1.611	1.613	1.615	1.617	1.619
6.	$(NH_4)_6 Mo_7O_{24}$	1.608	1.610	1.612	1.614	1.616	1.618	1.620	1.622	1.624	1.626
7.	$(NH_2)_2 CO$	1.771	1.773	1.775	1.777	1.779	1.781	1.783	1.785	1.787	1.789
8.	Control	1.340	1.342	1.344	1.346	1.348	1.350	1.352	1.354	1.356	1.358

soaking of the mycodeteriorated seeds of mustard Pusa 256 in chemicals is an attempt to repair the damage caused to the physiology and biochemistry the seed and seedlings.

Many micronutrients are prosthetic group of enzymes and some act as their activators. Also, these are irreplaceable. The role of mineral matter in plant growth has been emphasized[21] in remote past, and in various levels of plant physiology and biochemistry has been discussed recently[22]. Many metabolic reactions responsible for cellular repair have been observed by soaking the aged seeds with some chemicals[23]. Soaking of Deccan 103, Ganga 5 and MMH 6 varieties of maize seed in KH_2PO_4 increased the vigour index, and in NH_4NO_3, KH_2PO_4 and Gibberellic acid accelerated the rate of growth significantly. Recently soaking of wheat, gram and mustard seeds deteriorating due to *Aspergillus flavus*, *A. niger* and *Fusarium moniliforme* in IAA, KI, KNO_3, KH_2PO_4, NH_4NO_3 and $ZnSO_4$ has resulted in increased vigour and growth of seedlings and biochemical contents therein as estimated presently[11].

The chemicals soaked by the seeds are expected to act in the same way as absorbed by the soil and probably make up the essential minerals either absent there or are inadequately available. An exhaustive account of the role of mineral nutrition of crops has been documented on the growth and productivity[24]. Treatment to the seeds of cowpea and maize with domestic waste resulted in accelerated activity of hydrolytic enzymes dissolving reserve food of the seeds, their higher germination and better seedling stand[25]. Increase in TC content in the seedlings even by treatment to badly deteriorated seeds is highly expected to raise the level of TSS and subsequent metabolic reactions imparting increase in the dry weight of the seedlings. Similarly, higher activity of NR and UR due to treatment might raise the level of ammonia for the synthesis of compounds such as amino acids and subsequently the amides, proteins, nucleic acids etc. All the chemicals used for invigoration proved to regulate the physiological activity raising the levels of biochemicals essentials for the metabolism.

The seeds deteriorating due to fungi leach more cations and anions[26] due to dysfunction of the plasmamembrane which, too, can be compensated by invigoration treatment. $MgSO_4$, $(NH_4)_6Mo_7O_{24}$ and $(NH_2)_2CO$ raised the level of TC probably providing nitrogen and magnesium for regulating the synthesis of chlorophylls. Augmented activity of NR and UR has been observed to raise the level of amino acid in vegetative part of wheat and protein in seed[8]. Phosphorus is inseparable part of nucleic acids, ATP, NADP, Co-enzymes for photosynthesis, nitrogen, carbohydrate and fat metabolism. K^+ is intimately involved in stomatal opening and as activator in synthesis of peptides. Fe^{++} has affect on thylakoid membrane composition, photosystem I and II and fat metabolism. Cu^{++} is a part of many oxidative enzymes[26]. IAA, a growth promoting harmone, is involved in cell division, and is destroyed in the seedlings raised from the seeds deteriorating by fungi due to excited IAA oxidase[27] activity. The treatment with IAA is expected to compensate the loss. $(NH_2)_2CO$ and $(NH_4)_6Mo_7O_{24}$ seem to provide nitrogen to the seedlings. The later may provide molybdenum also essential for the physiological functions of plants.

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