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Impact of GA_3 encapsulated nanosilica on maize seed viability

Vinay Kumar Chourasiya[a], Prabha Shankar Shukla[b], Chhotey Lal Maurya[c], Birendra Prasad[b], Rakesh Choudhary[d], Deepankar Pandey[e] & Paras Kushwaha[c]

[a]Department of Seed Science and Technology, [b]Department of Genetics and Plant Breeding,

College of Agriculture, G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand 263 145, India

[c]Department of Seed Science and Technology, College of Agriculture, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur 208 002, India

[d]Genetics and Plant Breeding, College of Agriculture, Rani Lakshami Bai Central Agriculture University, Jhansi 284 003, India

[e]National Seed Association of India, New Delhi-110001

E-mail: vc3949@gmail.com

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The nanoparticles have the potential ability of the passing cell membrane because of their size in nano meters. The SiO_2 nanoparticles are one of the major and frequently used engineered oxide nanoparticles. In present investigation, the potential effect of SiO_2 (10-20 nm) nanoparticles on maize seed viability were studied. We observed quick result of seed viability as well as increases in seed viability percentage in presence of Gibberellic acid (GA_3) encapsulated silica nanoparticles. Three concentrations (0.5, 0.2 and 0.1%) of tetrazolium salts were used for staining of living tissue. The seeds preconditioned in 150 ppm GA_3 encapsulated nanosilica and tested in 0.5% tetrazolium salt gave maximum viable seeds which were due to the increased availability of GA_3 and showed quick staining over 0.2% and 0.1% concentration. Among the varieties, var. Navin imbibed in 150 ppm gibberellic acid encapsulated nanosilica showed a higher value of viable seed under 0.5% tetrazolium solution due to better availability of GA_3. Gibberellic acid enhances metabolic activity of seed by secretion of α-amylase enzyme which is important for quick staining of embryonic tissue.

Keyword: GA_3-Gibberellic Acid, Nanosilica, Seed, Tetrazolium, Viability

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Advance farming with its predisposition for innovation and exactness, requests that every seed ought to promptly grow and create energetic seedling making sure higher productivity. The seed has always been an important key factor in determining crop productivity and ensures food security to ever growing population. Seed quality is a fundamental trait for crop production and food security, especially during the expanding vulnerability because of environmental changes. Modern crop development and agricultural science also verify that we will not have an optimum crop yield without good quality seeds. Maize is one of the foremost necessary cereal crops on the planet and contributes to food security in most developing countries. Globally, maize is known as "Queen of Cereals" due to its highest yield potential among the cereal crops. In India, maize is third most important staple food crop after rice and wheat and maize adds approximately 9% in the domestic food basket and more than Rs. 100 billion to the present prices of agricultural GDP apart from the fact that it also generates employment more than 100 million man-days in agricultural and industrial sectors[3].

Nanoparticles are atomic or molecular aggregates of a large and flexible group of materials ranging from 1 to 100 nm[4]. The functionalization of nanoparticles with chemicals and organic/inorganic molecules could lead to the growth of products that can be used to address several issues linked to seed germination, intelligent targeted and long-term supply of agrochemicals such as growth regulators/fertilizers /pesticides and sensors for the fast identification of contaminants on-field. Nanosilica ($nSiO_2$) is a critical metal oxide that covers all significant scientific and technological areas, including industrial, electronics, agricultural and biomedical applications[6]. It has gained larger attention owing to its extremely reactive

*Corresponding author

surface-to-volume magnitude relation property. The introduction of nanoparticles into plants might need vital impact and so, it may be used for agricultural applications for higher growth and yield. An earlier study showed that the addition of nanosilica in soil enhanced maize (*Zea mays* L.) growth[13].

Lakon[9] established that all living cell of the seed which respire can reduce a colorless solution of 2,3,5-triphenyl tetrazolium chloride (TZ) or bromide into a red coloured compound called formazan. TZ has been widely accepted and reliable among chemical seed viability tests. The evaluation of seed viability of maize seeds is essential to determine establishment for successful crop production[1]. Keeping the note of above points in addition the beneficial effects of the nanosilica on growth regulator delivery in plant/seed cell, germination and seedling growth further investigation is needed to fully explore the nano-invigoration effect on seed quality parameters. The principal objective of this study was to determine whether the preconditioning solutions of different bioactive chemicals could be more effective in determining viability test of maize.

Materials and Methods

Varieties and treatments

An experiment was conducted on six maize varieties viz., V_1-Amar, V_2-PSM-3, V_3-D-765, V_4-Tarun, V_5-Sweta and V_6-Navin. Genetically pure seeds of these varieties were procured from Norman E. Borlaug Crop Research Centre, Pantnagar (Uttarakhand), India. The laboratory experiment is conducted in Biophysics and Nanotechnology Unit, College of Basic Science and Humanities, G. B. Pant University of Agriculture and Technology, Pantnagar (Uttarakhand), India and silica nanoparticles ($nSiO_2$), gibberellic acid (GA_3) and methanol were utilized to conduct the experiments. The solution of nanosilica with encapsulation of gibberellic acid was prepared and different concentrations were maintained according to the treatment requirements to conduct experiment. The seeds were subjected to various treatments viz., T_0-Control, T_1-8 g/l nano silica, T_2-100 ppm GA_3, T_3-8 g/l nano silica+100 ppm GA_3, T_4-8 g/l nano silica+125 ppm GA_3, T_5-8 g/l nano silica+150 ppm GA_3 and T_6-8 g/l nano silica + 100 ppm PEG, in replicated trials. The instruments used, procedure of synthesis of mesoporous nano-silica pellets and loading of gibberellic acid with the nanosilica is elaborated under the following heads:

Instruments

The zeta potential and size of the mesoporous nanosilica and urea with GA_3 encapsulated silica pellet were confirmed by dynamic light scattering and laser Doppler principles, respectively using Microtrac Nanotrac Wave II (Microtrac Instruments) with a scattering angle of 180°. The absorption spectra were recorded in the range of 254 nm by UV–Vis spectrophotometer UV-2600 (SHIMADZU Technologies). All samples were diluted 5-fold in distilled water before the treatment given for the data recording. The Fourier-transform infrared spectroscopy (FT-IR) study was done using a Nicolet iS50FT-IR spectrometer (Thermo Scientific, USA) and the spectra was measured between 4000 and 400 cm−1 in Attenuated Total Reflectance (ATR) for nanosilica characterization and 4000 and 720 cm-1 in Omni-Cell/Traditional Liquid Transmission Cell holder (ZnSe) for liquid sample characterization.

Synthesis of mesoporous nano-silica pellets

Firstly, 800 mg mesoporous nano-silica was weighed by the electronic balance and it is rinsed by adding 100 mL methanol and stirred by Coring stirrer (Model PC-420D) for 30 min at 320 rpm. The solution was centrifuged by Remi centrifuge (model no. C-24BL) for 5 min at 5000 rpm. The supernatant was removed with the help of micropipette from the tube and the process is repeated thrice and at last precipitate materials (pellet) was used to treat the seeds with and without gibberellic acid loading.

Encapsulation of GA_3 on the mesoporous nano-silica

The gibberellic acid was stabilized with urea and non-ionic adjuvant to overcome the problem of denaturation. The collected precipitated material (pellet) was added in the 100 mL methanol solution with the varying concentration of gibberellic acid (stabilized with urea and non-ionic adjuvant) such as 100 ppm, 125 ppm and 150 ppm. The different concentration of a mesoporous nano-silica with gibberellic acid solution in the beaker was kept on a stirrer for 24 h at 150 rpm. After, 24 h the solution is centrifuged for 5 min at 5000 rpm. Then, supernatant was removed from the centrifuge tube and pellet was collected for further seed invigoration treatment. The prepared pellet (GA_3) loaded mesoporous nano-silica) was used to prepare final solutionin distilled water (100 mL) for the conditioning of maize seeds prior to tetrazolium test.

Preconditioning of seed and viability test

The seeds of different maize varieties were preconditioned overnight (12 h) in the GA_3 encapsulated, nonencapsulated nanosilica and gibberellic acid solution and dried under shade to maintain the proper moisture content. Further, treated seeds were used for the germination test and seed viability test. The three concentrations of tetrazolium chloride salt aqueous solution (0.1%, 0.2% and 0.5%) were prepared. The seed viability test using tetrazolium was conducted. The procedure laid down by International Seed Testing Association (ISTA) was followed.

After the preconditioning of seed with nanosilica and gibberellic acid for a prescribed period (12 h) each seed was bisected longitudinally through the center of the embryo so that each half had a part of the plumule and the radicle. One half of each seed was then placed in four petri dish and poured 2,3,5-triphenyl tetrazolium chloride solution (0.1%, 0.2% and 0.5%) over the cut seeds until they were completely immersed. The petri dish was kept at 25°C for 30 min to proper staining of embryo and other essential structure of seeds. The sample (100 seeds) is satisfactorily stained when tissues develop interpretable staining characteristics and the analyst can 'sense' embryo condition. After proper staining the seed was removed from the tetrazolium solution, rinsed 2-3 times in water and then evaluated according to the staining pattern. During the course of observation on seed viability, seeds were kept in little water to prevent drying. For the clearing of solution 2-3 drops of lactophenol was added. The distinct staining of the vital parts of the embryo was evidence of ability to produce a normal seedling[8]. The viability percentage tested by staining of 2,3,5-Triphenyl Tetrazolium Chloride indicated the percentage of normal viable seeds.

Results and Discussion

Characterization

Particle Size Analyzer (PSA)

By particle size analyzer (PSA) technique, one may record data on the hydrodynamic diameter and the aggregation state of nanosilica prepared in methanol solution. In addition, the hydrodynamic diameter of nanosilica refers to the particle size with electrostatic potential radius around it. The size distribution of nanosilica and urea with Gibberellic acid encapsulated nanosilica pellet were measured by PSA and is shown in Figure 1a and b, respectively. The volume (42.4%) of nanosilica in Figure 1a is 273.7 nm and some volume (57.6%) is 985 nm in diameter. In Figure 1b, most of volume (85.2%) of nanosilica is 4340 nm and some volume (14.8%) of urea with GA_3 encapsulated nanosilica is 347 nm which increased to 3355 nm and 74 nm in the case of Figure 1a, respectively[18].

UV-Visible Spectrophotometer Analysis

In the last few decades, nanosilica and synthesis of nanosilica encapsulated with gibberellic acid has been an active research area in agriculture field because of their vital optical merits, which is strongly dependent on shape, size and nano-composition and can be analyzed with help of the optical instruments like UV-visible spectroscopy analysis. Even though, the absorption spectra were carried out using UV-visible spectrophotometer to check the stability of urea with GA_3 encapsulated nanosilica at different concentrations like 100, 125 and 150 ppm (Fig. 2). Figure 2 shows the maximum absorption peak of urea with Gibberellic acid encapsulated nanosilica pellet at different concentration without adjuvant (black line) and minimum absorption peak of urea-gibberellic acid encapsulated nanosilica pellet at different concentration with adjuvant (red line)[18].

FT-IR spectrometer

Figure 3a presents the FT-IR results of nanosilica. The FT-IR spectra of nanosilica sample show large bands of O-Si-O stretching at 1,079.81 and 793.67 cm^{-1}. The bands at 3365.07 and 1634.28 cm^{-1}

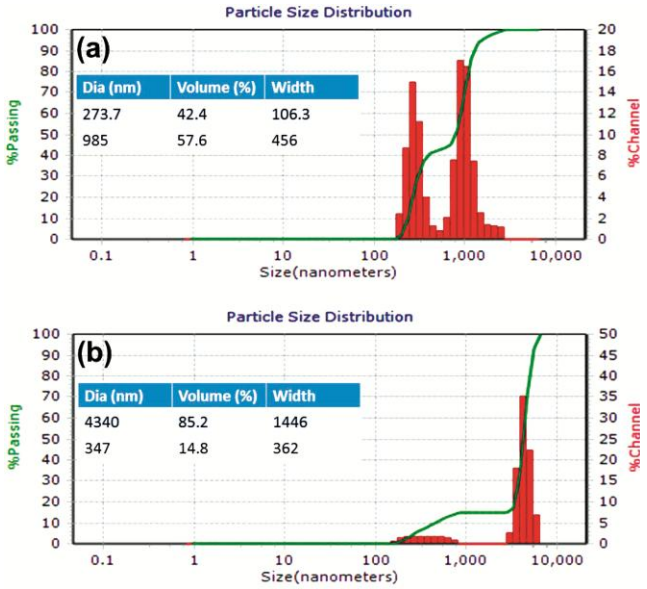

Fig. 1 — PSA spectra analysis (a) mesoporous nanosilica (b) Urea with GA_3 encapsulated mesoporous nanosilica

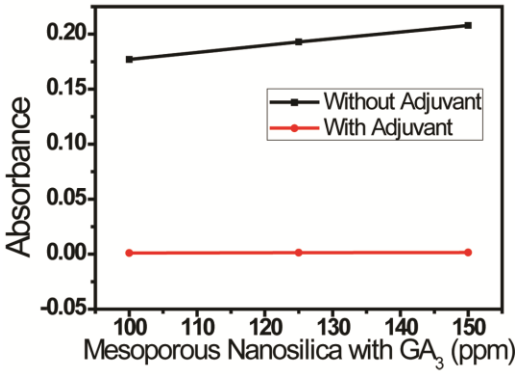

Fig. 2 — UV-Vis spectra analysis of urea with GA3 loaded nanosilica pellet without adjuvant (black line) and with adjuvant (red line)

correspond to the O-H stretching and bending vibrations as shown in Figure 3a. Furthermore, the FT-IR spectrum of urea (black line), urea with gibberellic acid (red line) and urea with gibberellic acid encapsulated nanosilica (blue line) is shown in Figure 3b which also shows the C=O stretching frequency at 1681 cm^{-1}. The N–H stretching and vibrational frequencies show at 3433 cm^{-1} and 1606 cm^{-1}, respectively. In addition, the C–N stretching frequency shows at 1455 cm^{-1}. Compared with urea, the conjugate of urea- gibberellic acid does not appear a peak in red line at 1606 and 1681 cm^{-1}, which could be ascribed to the amide bond like the CO-NH stretch. Furthermore, another characteristic peak of gibberellic acid at 1752 cm^{-1} corresponded to the stretch of carboxyl group expressively lost in the FT-IR spectrum of urea- gibberellic acid[20]. The FT-IR results showed that the carboxylic groups of gibberellic acid reacted with the NH_2 of urea. Furthermore, O-H stretching and bending vibrations peaks (blue line) do not appear due to reaction of urea with gibberellic acid as shown in Figure 3b.

Effect of GA_3 encapsulated nanosilica on seed viability

The effect of preconditioning treatments with gibberellic acid encapsulated nanosilica ($nSiO_2$) on seed viability and staining of living tissue are presented in Table 1 and depicted in Figure 4 to Figure 8. The results of seed viability are discussed below:

Effect of 0.5% tetrazolium solution

The treatments and varieties exhibited highly significant results ($p<0.01$) for seed viability. However, interaction was found to be non significant ($p>0.05$). The experiment exhibited wide range of variation among the maize varieties for viable seed

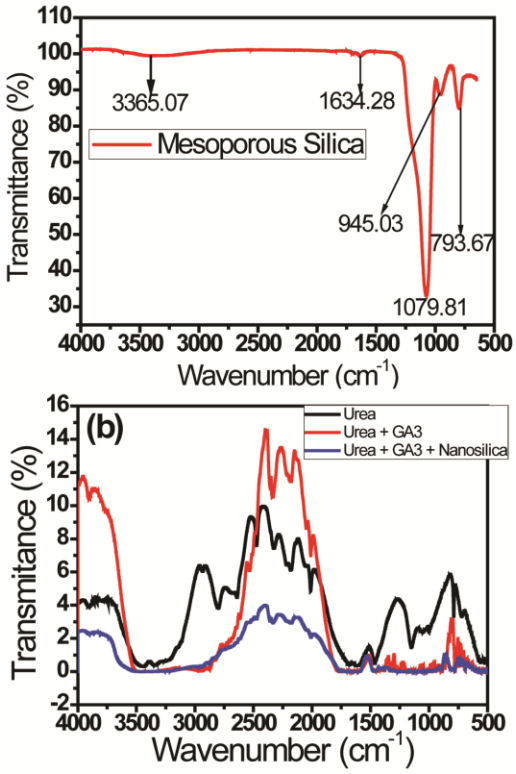

Fig. 3 — (a) FT-IR transmission spectra of nanosilica, (b) FT-IR transmission spectra of urea (black line), urea with gibberellic acid (red line) and urea with gibberellic acid encapsulated nanosilica (blue line)

percentage ranging from 81% to 86% at 0.5% concentration of tetrazolium solution (Table 1) and depicted in Fig. 9. The variety D-765 showed maximum viable seed (86%) among the all six varieties under study. The variation in viable seed with respect to the preconditioning treatments ranged from 74 to 90% which indicates significant differences in seed germination. However, among the preconditioning treatments, seed conditioned in 150 ppm gibberellic acid encapsulated nanosilica (T_5) exhibited maximum number of viable seed (90%) followed by 125 ppm gibberellic acid encapsulated nanosilica (88%). Among the interactions of varieties and preconditioning treatments, variety Navin imbibed in 150 ppm gibberellic acid encapsulated nanosilica solution (V_6T_5) produced numerically maximum viable seeds (94%) followed by V_6T_5 (92%). Results revealed that seed imbibed in 150 ppm gibberellic acid encapsulated nanosilica increases seed viability by 16% as compared to seed preconditioned in water. The data presented in Table 1 and Figure 4-8 elucidate that the minimum non-viable seed (14%) was observed in variety D-765. Among the preconditioning treatments, seed

Table 1 — Effect of silica nanoparticles (SiO_2) on seed viability of ten month aged seeds of maize varieties.

Viable seed percentage

Treatment	0.5% tetrazolium salt							0.2% tetrazolium salt							0.1% tetrazolium salt						
	V_1	V_2	V_3	V_4	V_5	V_6	Mean	V_1	V_2	V_3	V_4	V_5	V_6	Mean	V_1	V_2	V_3	V_4	V_5	V_6	Mean
T_0 (Control)	73	72	76	73	72	77	74	71	73	77	75	73	77	70	70	70	75	73	71	79	73
T_1 (SiO_2)	77	76	84	80	77	82	79	75	78	85	80	77	82	74	74	78	83	80	73	79	78
T_2 (GA_3 100 ppm)	75	77	83	77	76	82	78	73	76	84	77	75	80	75	75	75	84	77	76	79	78
T_3 (SiO_2 + GA_3 100 ppm)	82	85	88	80	83	85	84	82	85	88	82	83	87	81	81	83	87	81	81	84	83
T_4 (SiO_2 + GA_3 125 ppm)	86	87	91	89	84	90	88	86	88	91	88	85	91	81	81	90	89	86	83	88	86
T_5 (SiO_2 + GA_3 150 ppm)	91	88	92	89	88	94	90	91	90	93	90	89	93	89	89	90	92	88	86	89	89
T_6 (SiO_2 + PEG 100 ppm)	83	82	87	80	78	82	82	83	82	88	80	79	83	80	80	81	88	79	76	80	81
Mean	81	81	86	81	80	84	82	80	82	87	82	80	85	78	78	81	85	81	78	83	81
CV (%)	3.875							3.066							3.565						
	SEm (±)			CD (5%)			p value	SEm (±)			CD (5%)			p value	SEm (±)			CD (5%)			p value
Variety	0.695			1.955			2.01×10^{-27}	0.552			1.552			2.29×10^{-35}	0.630			1.772			1.89×10^{-29}
Treatment	0.751			2.112			7.2×10^{-9}	0.596			1.677			1.38×10^{-14}	0.681			1.914			1.65×10^{-12}
Variety X Treatment	1.839			5.173			0.702	1.460			4.107			0.555	1.667			4.689			0.109

Dead seed percentage

Treatment	0.5% tetrazolium salt							0.2% tetrazolium salt							0.1% tetrazolium salt						
	V_1	V_2	V_3	V_4	V_5	V_6	Mean	V_1	V_2	V_3	V_4	V_5	V_6	Mean	V_1	V_2	V_3	V_4	V_5	V_6	Mean
T_0 (Control)	27	28	24	27	28	23	26	29	27	23	25	27	23	26	30	30	25	27	29	21	27
T_1 (SiO_2)	23	24	16	20	23	18	21	25	22	15	20	23	18	20	26	22	17	20	27	21	22
T_2 (GA_3 100 ppm)	25	23	17	23	24	18	22	27	24	16	23	25	20	22	25	25	16	23	24	21	22
T_3 (SiO_2+GA_3 100 ppm)	18	15	12	20	17	15	16	18	15	12	18	17	13	16	19	17	13	19	19	16	17
T_4 (SiO_2+GA_3 125 ppm)	14	13	9	11	16	10	12	14	12	9	12	15	9	12	19	10	11	14	17	12	14
T_5 (SiO_2+GA_3 150 ppm)	9	12	8	11	12	6	10	9	10	7	10	11	7	9	11	10	8	12	14	11	11
T_6 (SiO_2+ PEG100 ppm)	17	18	13	20	22	18	18	17	18	12	20	21	17	18	20	19	12	21	24	20	19
Mean	19	19	14	19	20	16	18	20	18	13	18	20	15	18	22	19	15	19	22	17	19
CV (%)	3.875							3.066							3.565						
	SEm (±)			CD (5%)			p value	SEm (±)			CD (5%)			p value	SEm (±)			CD (5%)			p value
Variety	0.695			1.955			2.01×10^{-27}	0.552			1.552			2.29×10^{-35}	0.630			1.772			1.89×10^{-29}
Treatment	0.751			2.112			7.2×10^{-9}	0.596			1.677			1.38×10^{-14}	0.681			1.914			1.65×10^{-12}
Variety X Treatment	1.839			5.173			0.702	1.460			4.107			0.555	1.667			4.689			0.109

V_1-Amar, V_2-PSM-3, V_3-D-765, V_4-Tarun, V_5-Sweta and V_6-Navin

conditioned in 150 ppm gibberellic acid encapsulated nano silica recorded minimum nonviable seed (6%). The treatment with tetrazolium salt solution 0.5% concentration showed quick staining of seed preconditioned in different gibberellic acid encapsulated nanosilica as compared to seed imbibed in water which may be due to better availability of gibberellic acid[13,17]. Encapsulation of gibberellic acid to nanosilica accelerates the availability of gibberellic acid to living embryonic tissue during the staining

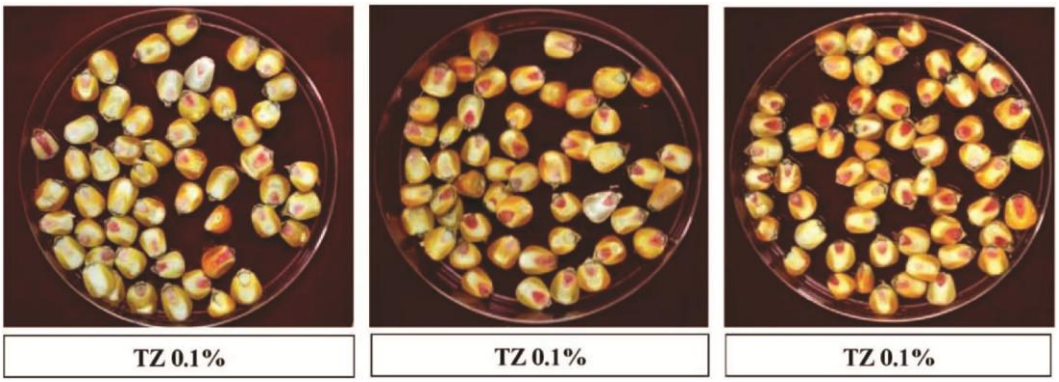

Fig. 4 — Effect of tetrazolium salt concentration on staining intensity of water imbibed maize seeds

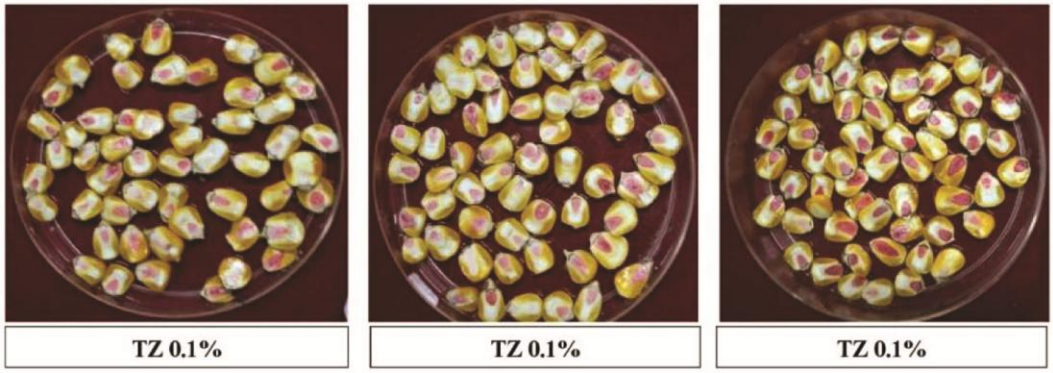

Fig. 5 — Effect of Tetrazolium salt concentration on staining intensity of maize seeds imbibed in 100 ppm GA_3 loaded nanosilica

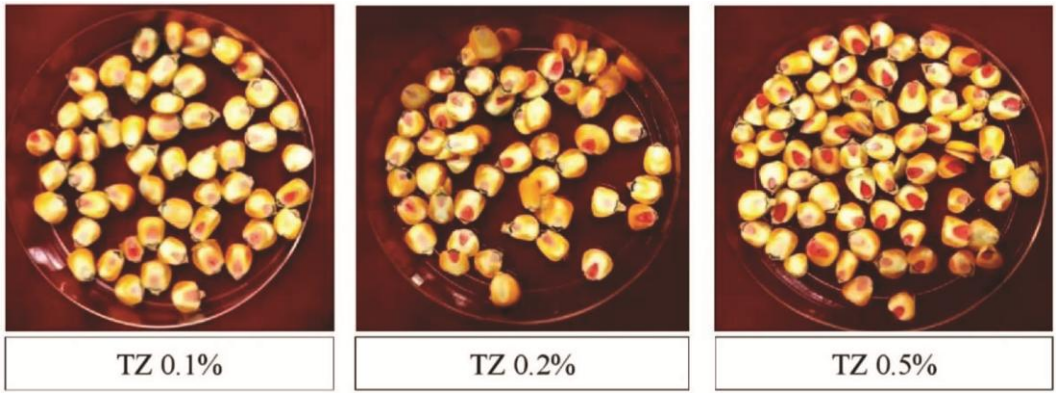

Fig. 6 — Effect of Tetrazolium salt concentration of staining intensity of Amar variety seeds imbibed in 150 ppm GA_3 loaded nanosilica

that enhances the viability of the seed.

Effect of 0.2% tetrazolium solution

The data given in Table 1 showed that there were highly significant ($p<0.01$) variations due to different preconditioning treatments and varieties with respect were observed on seed viability. While, the interaction of treatment x variety did not show ($p>0.05$) differences. Experiment exhibited that maize varieties imbibed in different concentration of gibberellic acid and gibberellic acid encapsulated with nanosilica was varied for viable seeds from 80 to 87%. Among the varieties, D-765 had maximum viable seeds (87%). The results presented in Table 1 and Fig. 2 disclose that nano-conditioning treatments varied for viable seeds from 70 to 89% being the maximum in seed preconditioned in 150 ppm gibberellic acid encapsulated nanosilica (89%) which is 19% higher as compared to control. The variety D-765 and Navin preconditioned in 150 ppm gibberellic acid encapsulated nanosilica exhibited maximum viable seeds (93%). The results revealed that minimum nonviable seed per cent (13%) was reported in variety D-765, followed by variety Navin (15%). Among the preconditioning treatments, 150 ppm

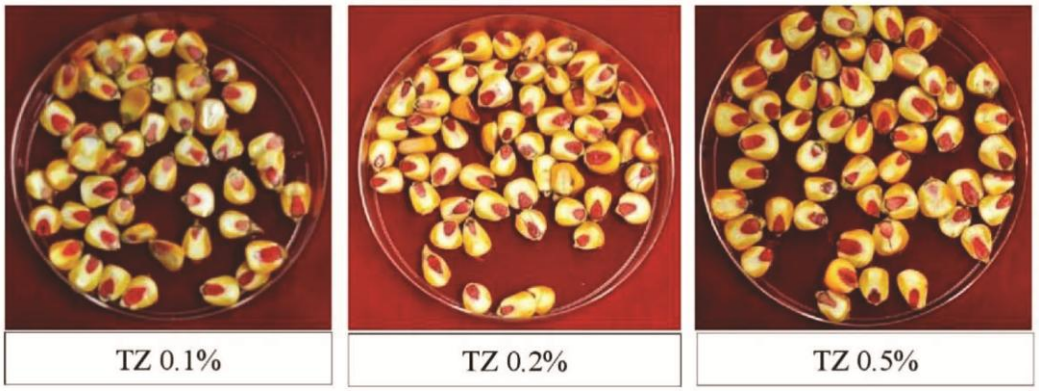

Fig. 7 — Effect of Tetrazolium salt concentration on staining intensity of PSM-3 variety seeds imbibed in 150 ppm GA_3 loaded nanosilica

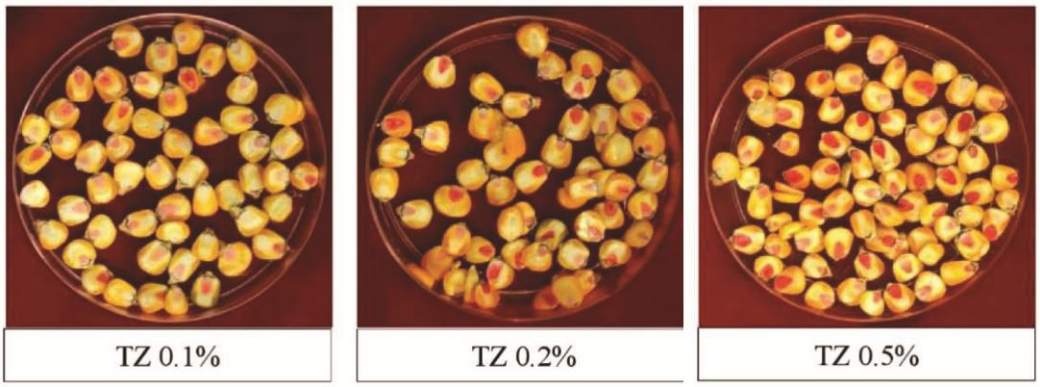

Fig. 8 — Effect of Tetrazolium salt concentration on staining intensity of D-765 variety seeds imbibed in 150 ppm GA_3 loaded nanosilica

gibberellic acid encapsulated nanosilica showed minimum percent of nonviable seeds (9%) followed by 125 ppm gibberellic acid encapsulated nanosilica (12%). It was found that 150 ppm gibberellic acid encapsulated nano silica showed 17% less nonviable seed percent as compared to control (26%). Results also exhibited that maize variety D-765 and Navin imbibed with 150 ppm gibberellic acid encapsulated nano silica registered minimum nonviable seed (7%).

Effect of 0.1% tetrazolium solution

Varieties and seed preconditioning treatments showed highly significant differences ($p<0.01$) for seed viability. The interaction effect between varieties and seed invigoration treatments, by contrast, showed no statistical differences ($p>0.05$) for seed viability. The percentage of viable seeds among the varieties varied from 78% - 85% (Table 1). The variety D-765 showed maximum viable seed (85%) which is 7% more as compared to least performed varieties Amar and Sweta. Among the preconditioning treatments viable seed percent varied from 73% to 89% (Table 1). The seeds preconditioned in 150 ppm gibberellic acid encapsulated nanosilica had maximum viable seed percent (89%) which is 16% higher as compared to control. Results exhibited that variety D-765 preconditioned with 150 ppm gibberellic acid encapsulated nano silica had maximum viable seed percent over the other interaction. The results also revealed that varieties D-765 recorded minimum per cent of nonviable seeds (15%) followed by variety Navin (17%). The preconditioning treatments showed minimum per cent of nonviable seed (11%) recorded in preconditioning treatments with 150 ppm gibberellic acid encapsulated nanosilica, followed by 125 ppm gibberellic acid encapsulated nanosilica (14%), whereas maximum nonviable seed percent was found in untreated control (22%). The var. D-765 preconditioned in 150 ppm gibberellic acid encapsulated nanosilica solution resulted minimum nonviable seed (8%).

Overall, it can inferred that seeds preconditioned in gibberellic acid encapsulated nanosilica offer quick staining as compared to water imbibed seeds owing to improved nanosilica accessibility of gibberellic acid in embryonic portion of seed[14]. The fastest and most

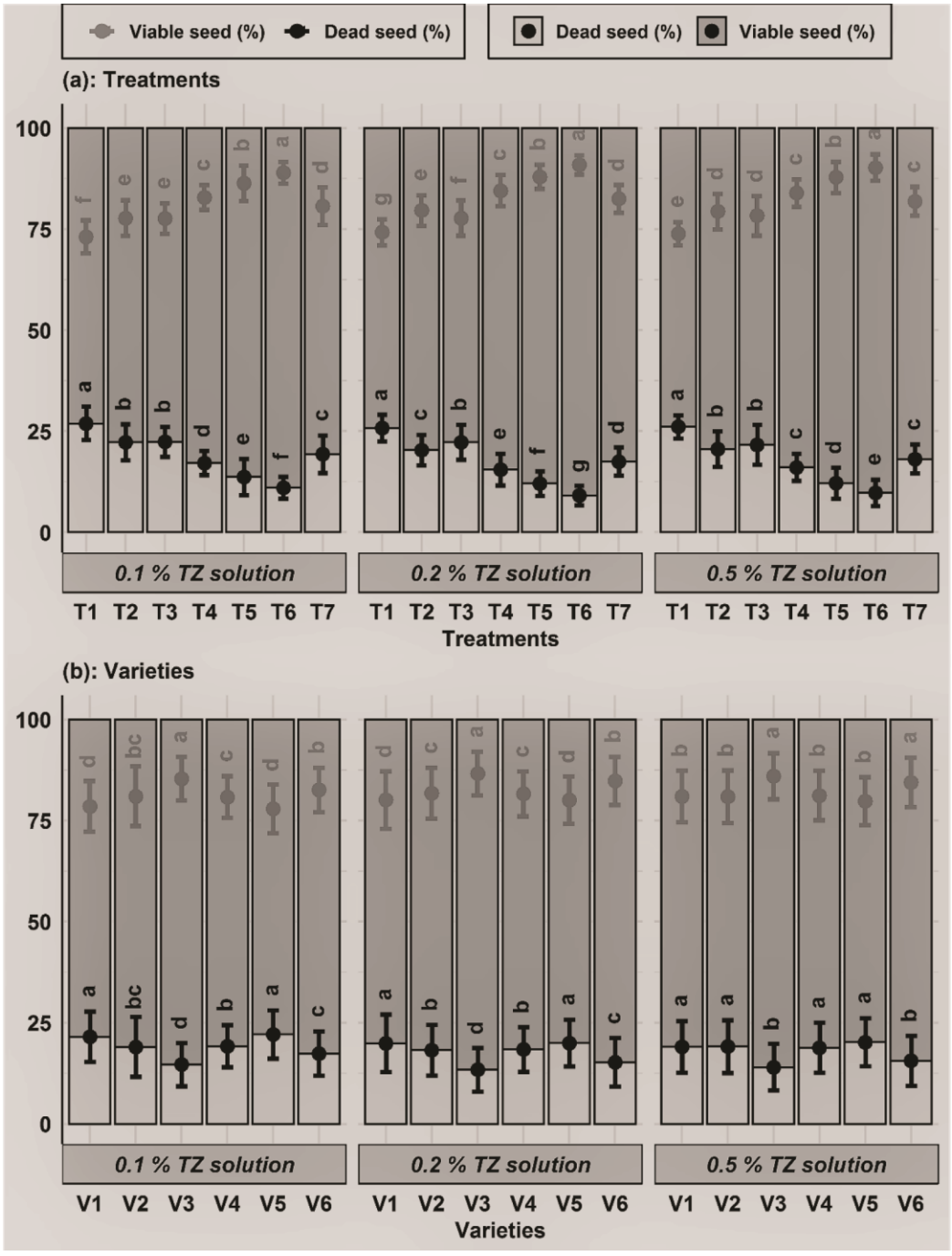

Fig. 9 — Graphical Representation of Tetrazolium Salt on staining intensity of Gibberellic acid encapsulated maize varieties seeds

uniform staining of embryo was observed in seed preconditioned in 150 ppm gibberellic acid in all three tetrazolium solution (0.1%, 0.2% and 0.5%). The nano-SiO_2 enhanced seed germination by increasing the accessibility of nutrients, water absorption enhanced enzymatic activity, which is the primary cause of rapid seed staining[9,13,16]. Difference in seed viability of different varieties of maize could be due to its genetic makeup and bioactive of enzyme needed for staining of embryo. The accessibility of gibberellic acid stimulates the synthesis, activation and secretion of hydrolytic enzymes, primarily α-amylase, releasing vital sugars and amino acids for embryo growth[10]. The colorless tetrazolium salt is reduced by activity of dehydrogenase enzyme to produce red colored product formazan. The staining

pattern of the red formazan is an indicative of active dehydrogenase respiratory enzymes[4,12]. The GA_3 encapsulated nanosilica enhances seed viability & germination of maize which increase plant population in field and ultimately increase seed yield of maize and other cereal crops.

The present study can be concluded that maize seed preconditioned in 150 ppm GA_3 encapsulated nanosilica and staining in 0.5% tetrazolium salt solution gave more precise results as compared to & other treatments. Overall results showed that the presence of silica nanoparticles in different GA_3 encapsulating concentrations affects the staining of maize seed. Maize seed preconditioned in 150 ppm GA_3 encapsulated nanosilica and staining in 0.5% tetrazolium salt solution yield faster outcomes compared to water preconditioned seeds. Among the maize varieties D-765 showed higher value of percentage viable seed under 0.5% tetrazolium solution which might be enhanced secretion α-amylase for increasing metabolic activity of maize seeds. Silica nanoparticles played key role in GA_3 delivery in seed embryo which is important for quick staining. In the future GA_3 encapsulated nanosilica is going to play a very important role in seed germination, increasing plant population and seed yield as well as grain harvest.

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Conflict of Interest

The authors declare that they have no competing interests.

Authors' Contributions

VKC conceived, designed and conduct the research. RC and DP contributed to the analysis of data and statistics. PSS and BP supervised the research and suggested valuable comments. PK contributed in preparing manuscript. CLM guided in preparation of manuscript and reviewed and edited the manuscript and suggested critical comments. All authors read and approved the manuscript.

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