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Exogenous application of aba and green synthesized copper nanoparticles for quantification of drought tolerant biomolecules in Calli of *Sorghum bicolor*

Kiran, Bharti Yadav, Rishabh Shrivastava Ronin, Manas Mathur, Garima Vaishnav, RS Sain, Parth Jhorar, Arbaj Pathan and Rakesh Kr Prajapat

Abstract
The of variety CSV-17 of *Sorghum bicolor* seeds culture on different concentrations and combination of 2, 4-D, Kn, BAP, IAA, IBA, NAA was examined. The best callus volume were observed in 2.5 + 0.5 mg/L (2, 4-D and Kinetin). Copper nanoparticles (Cu NPs) were synthesized using the leaves of *Moringa oleifera* as reducing and capping agent and further characterized through UV-Visible spectroscopy and SEM. For subculturing, the fresh, viable and actively growing and proliferating calli pieces were weighed (1.0 g) and cultured on solid callus proliferation medium (MS) consisting of 2,4-D and Kn (2.0 + 0.5 mg/l)] with various doses of ABA and polyethylene glycol (control, 2, 6, 10 mg/L and control, 1.0, 1.5, 2 mg/L) respectively. Another media (MS) synergetically supplemented with Polyethylene glycol, ABA and copper nanoparticles doses (control, 1.0 + 2 + 5 mg/L; 1.5 + 6 + 10 mg/L; 2 + 10 + 20 mg/L) respectively and harvested for estimation of glycine-betaine and proline quantification after 6th week. Positive effects were more considerable at its moderate dose ABA + PEG (6 + 1.5 mg/L); Cu-NPs + ABA + PEG (10 mg/L + 6 mg/L + 1.5 mg/L) and introducing it as the best treatment to ameliorate sorghum performance especially accumulation of proline and glycine betaine under drought stress. ABA and CuNps + ABA application at its higher dose (10; 10 + 20 mg/L) respectively demonstrated toxicity by negative impacts on accumulation of proline and glycine betaine. Plantlet regeneration occurred on MS containing BAP + Kn (2.0 + 0.3 mg/L); BAP + NAA (2.0 + 0.2 mg/L); BAP + Kn + NAA (2.1 + 1.0 + 0.3 mg/L) for shooting and IBA + NAA (3.5 + 0.4 mg/L) for rooting. In conclusion, the positive response of sorghum to ABA and ABA + CuNPs under drought stress conditions cause to consider that ABA and CuNps + ABA as potential novel plant growth promoting and stress protecting agent.

Keywords: Sorghum, drought stress, green synthesis, ABA, copper nanoparticles, proline, glycine betaine

Introduction
Nanoparticles are extremely small particles with a size range of 1–100 nm. Applications of nano SiO2, TiO4, Zeolite, and ZnO promote seed germination in crop plants [1]. In a few studies, nanoparticles were demonstrated to have the capacity to penetrate the seed coat and stimulate water absorption and utilization, which activates the enzymatic system and subsequently increases the germination rate [2]. Cu is a very significant naturally occurring plant micronutrient that participates in a variety of plant metabolic pathways as both an enzyme and a co-enzyme. By the metabolism of poly phenols, copper plays a critical role in lignification of cell walls, which affects the water balance in plants. Moreover, it helps with respiration, protein metabolism, carbohydrate synthesis, and photosynthesis [3, 4]. Copper is also a key ingredient in the production of chlorophyll biosynthesis [5].
Sorghum is regarded as one of the species that is most resilient to genetic modification and tissue culture regeneration, nevertheless. The main barriers to *in vitro* S. bicolor propagation are genotype-dependent poly phenol synthesis and tissue browning. Explants respond poorly as a result and regeneration rates are low. Nevertheless, numerous studies on the regeneration of sorghum using embryogenic callus from various explants have been published, including those using immature inflorescences [6, 7], immature embryos [8, 9], shoot tips, or meristems [10]. Because of their remarkable capacity for regeneration, young inflorescences and embryos are typically regarded as the best explants. Explants made from seeds are thus unquestionably more practical and appropriate [11, 12, 13].

One of the most often used methods for *in vitro* drought stress generation is the use of high molecular weight osmotic compounds, such as PEG. In order to imitate drought stress in cultured plant tissues, PEG6000, a non-penetrable and non-toxic osmotic material, is employed to lower the water potential of the culture medium [14].
ABA causes stomatal closure and lowers water loss through transpiration, increasing tolerance to environmental circumstances related to water stress [15, 16]. When exposed to various environmental conditions, such as salt, cold, or dehydration, ABA promotes gene expression [17].

Material and methods

The initial experiment was carried out at the Seminal Applied Sciences laboratory, Jaipur and some part completed at Genetics and Plant Breeding laboratory in Surseh Gyan Vihar University, Jaipur during 2022-2023 over a period of 6 months.

Callus initiation

Seeds of Sorghum variety CSV-17 were obtained from the IARI (Indian Agriculture Research Institute, Delhi). For sterilization, the seed were treated with Tween 80 (2-3 drops/100ml of water) for 5 minutes and rained with distilled water 2 or 3 time, disinfected with alcohol (70%) for two minutes again rained with distilled water three times. Then they exposed to commercial 0.1% aqueous $HgCl_2$ for 2 minutes followed by 3-4 times washings with distilled water to remove the traces of $HgCl_2$ in a laminar flow hood. The callus initiation medium contained MS salts and vitamins (Murashige & Skoog, 1962) [31] 30 g/L w/v sucrose and 0.8% Agar Agar was used for solidification of media. The pH of the medium was adjusted to 5.8 with 1N NaOH or 1NHCl before autoclave at 15 lb (121 °C) for 15-20 minutes. The effect of growth regulators was tested by fortifying media with different concentration and combination with each other of 2,4-D, Kn, BAP, IAA, IBA, NAA (Auxins and Cytokinins) as shown in Table 1 Medium without growth regulators was used as control. With the help of forceps seeds were inoculated in autoclaved media. Cultures were maintained dark and light conditions at 25±10C, while fluorescent tubes (using 40 watts) and incandescent lights (using 40 watts) provided 1200 lux of light.

Biological Reduction of copper nanoparticles

Fresh Moringa oleifera leaves were used to biologically reduced nanoparticles with the help of mortar and pestle. In order to synthesis copper nanoparticles, 0.01gm (2.0 mM) of copper sulphate was used as a precursor source. Overnight, let the solution sit. After incubation, colour started to emerge. The degree of colour intensity indicates the reduction of synthesized copper nanoparticles. The filtrate was taken in the eppendorf and it was centrifuged at 40 C, 10,000 rpm for 15 minutes. After centrifugation supernatant was discarded and pallet copper nanoparticles was collected and dried to remove moisture and thereafter processed for characterization for SEM and UV studies.

Table 1: Media preparation using different combination and concentration of PGRs for callus induction

Growth Hormones	Modified MS Media	Concentration (mg/L)
(i) Without Hormones MS media	MS_{00}	---
(ii) Callus Induction MS + 2,4-D + Kn	MS_1	1.5 + 0.5
	MS_2	2.5 + 0.5
	MS_3	3.0 + 0.02
	MS_4	3.5 + 0.02
	MS_5	4.0 + 0.5
MS + BAP + IAA	MS_6	1.0 + 0.2
	MS_7	3.0 + 3.0
MS + BAP + IBA	MS_8	1.0 + 0.2
	MS_9	3.0 + 3.0
	MS_{10}	3.0 + 4.0
MS + BAP + NAA	MS_{11}	1.0 + 2.0
	MS_{12}	1.0 + 0.2
MS + IAA + IBA	MS_{13}	1.0 + 0.3

Characterization of Copper Nanoparticles

The synthesized Copper nanoparticles were then characterized by UV-Vis spectroscopy and Electron microscopy to identify size of synthesized particles.

UV-Vis Analysis

UV-vis spectra of these aliquots (Cu nanoparticles) were monitored as a function of time of reaction on a Schimadzu 1601 spectrophotometer in 250–600 nm range operated at a resolution of 1 nm. For the analysis, 0.1 mL of the sample in a cuvette and was diluted to 2 ml with deionized water.

SEM (Scanning Electron Microscopy)

The SEM (Scanning Electron Microscopy) technique is used to ascertain the surface properties and size of nanoparticles. A thin layer of Cu was coated to make the samples conductive. The SEM metaphors were chronicled at 3,00,000 × magnification working at 10.00 keV. Scanning electron microscopic (Navo Nano SEM 450) was done at Malviya National Institute of Technology (MNIT), Jaipur.

Subculturing procedure under PEG stress treatment

Only the fresh, viable and actively growing and proliferating calli pieces were weighed (1.0 g) and cultured on solid callus proliferation medium [MS consisting of 2,4-D and Kn (2.5 + 0.5 mg/L)] and then transferred to fresh medium after every 2 week. Media was also supplemented with various doses of ABA and polyethylene glycol (control, 2, 6, 10 mg/L and control, 1.0, 1.5, 2 mg/L) respectively (Table 2). Another media synergetically supplemented with Polyethylene glycol, ABA and copper nanoparticles doses (control, 1.0 + 2 + 5 mg/L; 1.5 + 6 + 10mg/L; 2 + 10 + 20 mg/L) respectively (Table 3) and harvested for estimation of glycine- betaine and proline quantification after 6th week.

Table 2: Treatment of plant hormone along with various concentration of ABA for production of proline and glycine betaine

Prepared media (30 ml)	PEG Conc. (mg/L)	2, 4-D + Kn Conc. (mg/L)	ABA Conc. (mg/L)
Treatment 1	-	2.5 + 0.5	-
Treatment 2	1.0	2.5 + 0.5	2 mg
Treatment 3	1.5	2.5 + 0.5	6 mg
Treatment 4	2	2.5 + 0.5	10 mg

Table 3: Treatment of plant hormones along with various concentration of ABA and synthesised copper nanoparticles for production of proline and glycine betaine

Prepared media (30 ml)	PEG Conc. (mg/L)	2,4 D + Kn Conc. (mg/L)	ABA Conc. (mg/L)	ABA + CuNp Conc. (mg/L)
Treatment 1	-	2.5 + 0.5	-	-
Treatment 2	1.0	2.5 + 0.5	2 mg	2 mg + 5 mg
Treatment 3	1.5	2.5 + 0.5	6 mg	6 mg + 10 mg
Treatment 4	2	2.5 + 0.5	10 mg	10 mg + 20 mg

Relative growth rate index

At the transfer age of 2, 4, 6, and 8 weeks, the retained calli were routinely extracted. Every callus sample was collected, and growth indices (GI) were computed using fresh weights for each sample. RGR calculated by: [(Wf - Wi)/ Wi] / 100.

Quantification and estimation of glycine betaine and proline from *in vitro* culture

Free Proline content

According to [18] free proline content was measured spectrophotometrically. About 500 mg of fresh calli were homogenized in 3% (w/v) aqueous 5-sulfosalicylic acid, and the residue was removed by centrifugation at 10,000g for 10 min at 4 ℃. The supernatant (2 ml) was reacted with 2 ml each of acid ninhydrin and glacial acetic acid by incubating at 100C in a hot water bath for 1 h. The reaction was terminated in an ice bath and allowed to cool at room temperature. The reaction mixture was extracted with 4 ml toluene and mixed vigorously with a stirrer for 10–15s. The chromophore containing toluene was aspirated from the aqueous phase and warmed to room temperature. The optical density was measured at 520 nm (UV-1700 PharmaSpec, UV– Visible spectrophotometer; Shimadzu, Japan) using toluene as a blank. The amount of proline was determined from a standard curve using L-proline and expressed as µg of proline g-1 FW.

Glycine betaine (GB) analysis.

According to [19] accumulation of GB in response to drought tolerant was determined The fresh calli (500 mg) were ground in liquid nitrogen and the finely ground powder was mechanically shaken with 20 ml of demonized water for 16 h at 25 ℃. Samples were filtered, and filtrates (500 µl) were diluted (1:1) with 2N H2SO4. The extract was cooled in an ice water for 1 h and then mixed with 200 µl of I2-KI reagent (mixed with 20% potassium iodide and 15.7% iodine). The tubes were gently mixed and stored at 4C for 16 h followed by centrifugation at 10,000 g for 15 min at 00C. Periodide crystals were dissolved in 9.0 ml of 1, 2-dichloroethane, and after 2 h, absorbance was measured spectrophotometrically at 365 nm (UV-1700 Pharma Spec, UV–Visible spectrophotometer; Shimadzu). The GB content (µg g-1 FW) was determined from a standard curve prepared using glycine betaine (Sigma–Aldrich) as standard.

Plant regeneration from induced calli under PEG stress condition

To induce shoots and proliferate shoots we cultured best sub cultured callus on induction media (Murashige and Skoog (1962) [31] under condition: 25±2 ℃ 16-h photoperiod with white fluorescent lights 90 µmol m−2 s−1 and relative humidity of 70%.

Regenerated shoots were transferred onto rooting medium for root development when their heights reached over 3cm.

Hardening procedure

Plantlets were washed with distilled water to eliminate the remaining media from roots and to avoid further bacterial and fungal contamination due to media. Further, these plantlets were transferred to with autoclaved soil and vermin culture in 3:1 proportions. The plantlets were wrapped with polythene bags to retain humidity and watered with sterile, distilled water containing a 1:1 solution of ammonium nitrate and potassium nitrate. After the plants had stabilized and been acclimated, they were moved to the field from these containers while still at 25 ℃ and 16 hours of light. Table 4 shows modified media prepared using different combination and concentration doses of Growth Hormones.

Table 4: Modified media preparation using different combination and concentration doses of Growth Hormones for Plant regeneration

Growth Hormone	Modified MS media	Conc.(mg/L)
1. MS + BAP + Kn	MS_1	1.0 + 0.1
	MS_2	1.5 + 0.2
	MS_3	2.0 + 0.2
	MS_4	2.0 + 1.0
2. MS + BAP + NAA	MS_5	1.0 + 0.1
	MS_6	1.0 + 0.2
3. MS + Kn + IAA	MS_7	1.0 + 0.5
	MS_8	1.0 + 0.5
	MS_9	2.0 + 1.0
	MS_{10}	3.0 + 1.0
4. MS + BAP + Kn + NAA	MS_{11}	4.0 + 2.0
	MS_{12}	1.0 + 0.1 + 0.1
	MS_{13}	2.0 + 1.0 + 0.2
5. MS + IAA + IBA	MS_{14}	2.0 + 0.3 + 0.3
	MS_{15}	1.0 + 1.0
	MS_{16}	1.0 + 2.0
	MS_{17}	0.5 + 3.0
	MS_{18}	2.0 + 5.0
6. MS + IBA + NAA	MS_{19}	1.0 + 2.0
	MS_{20}	2.0 + 3.0
	MS_{21}	3.0 + 0.5
	MS_{22}	3.0 + 0.3
	MS_{23}	3.0 + 0.4

Statistical Analysis

The experiment was laid out in LSD (Latin Square Design). The data were analysed by one way analysis of variance (ANOVA) using the statistical software SPSS 16 and treatment means were compared by using Duncan's multiple

range test (DMRT) at $p \leq 0.05$. Data were expressed as mean ± standard error (SE).

Result

Callus induction from seed

Callus induction started after a week of germination of seeds (8-10 days). Colour of calli changed from off-white to lemon yellow during one month time period. Calli were soft and slimy in texture, but not very compact. Small globular structures also appeared on the surface of some calli. The callus grew slowly for the first four weeks, then proliferated more for the next two weeks, and ultimately, starting in the seventh week, the growth became stagnant and then started to diminish.

The media began to become brown around the eighth week, which may have been caused by phenols seeping out and phenolic compounds building up in the medium. The probability of the *Sorghum bicolor* callus tissue surviving and proliferating was higher during the fifth or sixth week as compared to 7th or 8th week calli (Figure 1). It was observed that best callus induction was achieved with 2.5 + 0.5 and 3.0 + 0.002mgL/L (2, 4-D + Kn). Calli cultured on the media containing both 2, 4-D and Kn proliferated well and showed totipotency. Thus, a medium supplemented with 2, 4-D 2.5 mg/L and 0.5 mg/L Kn was selected as the standard maintenance medium for sub-culturing totipotent calli. Higher levels of 2, 4-D (4.0 + 0.5 mg/L) were found to inhibit callus proliferation as shown in Table 5.

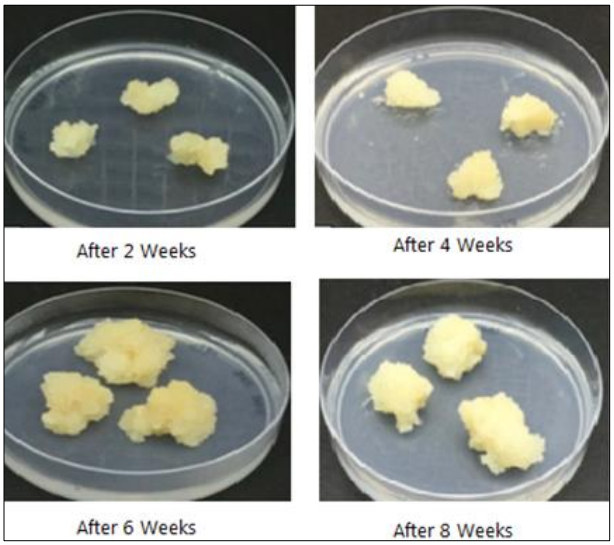

Fig 1: Callus Induction

Table 5: Result and analysis for callus induction using different combinations of PGRs concentration

Growth Hormones	Modified MS Media	Concentration (mg/L)	Explant used		
			Seeds		
			Biomass	Morphological Appearance	Nature of allus
(i)Without Hormones MS	MS_{00}	-	-	-	-
(ii) Callus Induction MS + 2,4-D + KN	MS_1	1.5 + 0.5	C +	BN	FG
	MS_2	2.5 + 0.5	C + + + +	BN	FG
	MS_3	3.0 + 0.02	C + + + +	BN	FG
	MS_4	3.5 + 0.02	C + + +	BN	FG
	MS_5	4.0 + 0.5	C + +	BN	FG
MS + BAP + IAA	MS_6	1.0 + 0.2	C +	BN	FG
	MS_7	3.0 + 3.0	C + + +	BN	FG
MS + BAP + IBA	MS_8	1.0 + 0.2	C + +	BN	FG
	MS_9	3.0 + 3.0	-	BN	FG
	MS_{10}	3.0 + 4.0	-	BN	FG
MS + BAP + NAA	MS_{11}	1.0 + 2.0	C + + +	GN	FG
	MS_{12}	1.0 + 0.2	-	GN	FG
MS + IAA + IBA	MS_{13}	1.0 + 0.3	-	GN	FG
C + /C + + /C + + + /C + + + + = Amount of callus produced, FG Fragile; GN-Green, BN- Brown, - = No response.					

Relative growth rate index

For the determination of fresh weight (FW), a defined quality of cultures were harvested on the 33rd day and then weighed. Dry weight (DW) was measured in these samples after being dried at 800C. RGR calculated by: [(Wf – Wi)/ Wi] / 100. The yield for 2^{nd}, 4^{th}, 6^{th}, 8^{th} week was 0.75, 1.10, 3.06, 2.65 respectively (Table 6)

Table 6: Growth index of calli in every two week

S. No.	Age of callus in (weeks)	Growth Index
1.	2	0.75
2.	4	1.10
3.	6	3.06
4.	8	2.65

UV-Visible Spectroscopy

By acquiring a spectrum with an invisible range of 250 nm to 650 nm, the existence of nanoparticles was established. The development of a specific absorption peak associated with Cu-NPs at 422–430 nm verifies the green method of nanoparticle synthesis as seen in Figure 2.

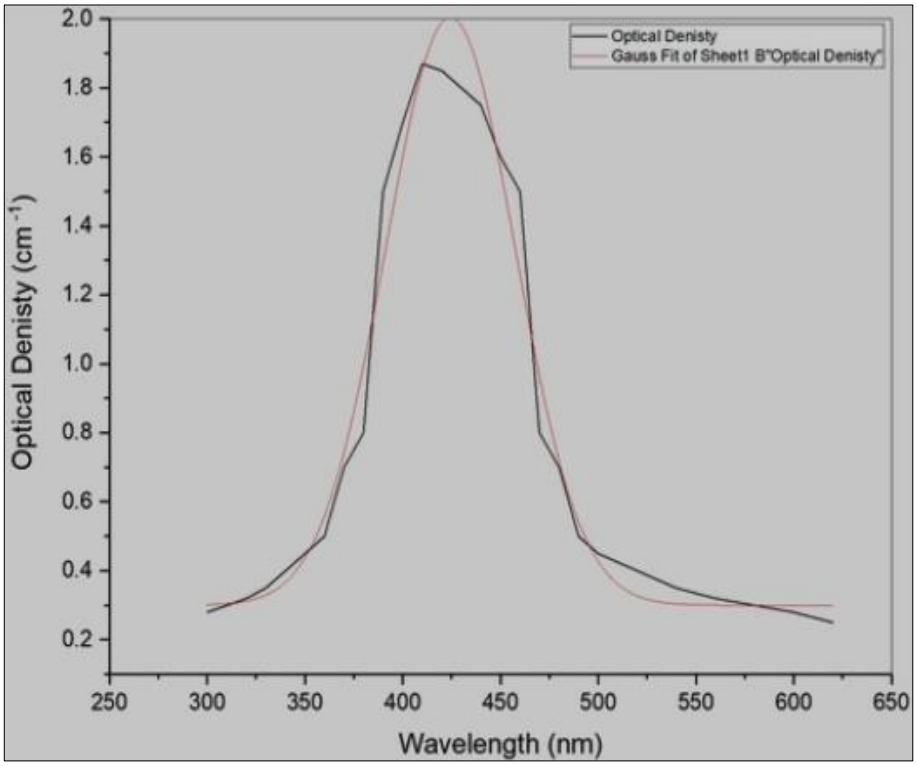

Fig 2: UV Spectra of Copper Nanoparticles

Scanning Electron Microscopy

The SEM (Scanning Electron Microscopy) technique is used to ascertain the surface properties and size of nanoparticles. The biosynthesized NP are probably very spherical and aggregated and size 80 nm as shown by SEM profiling (Figure 3).

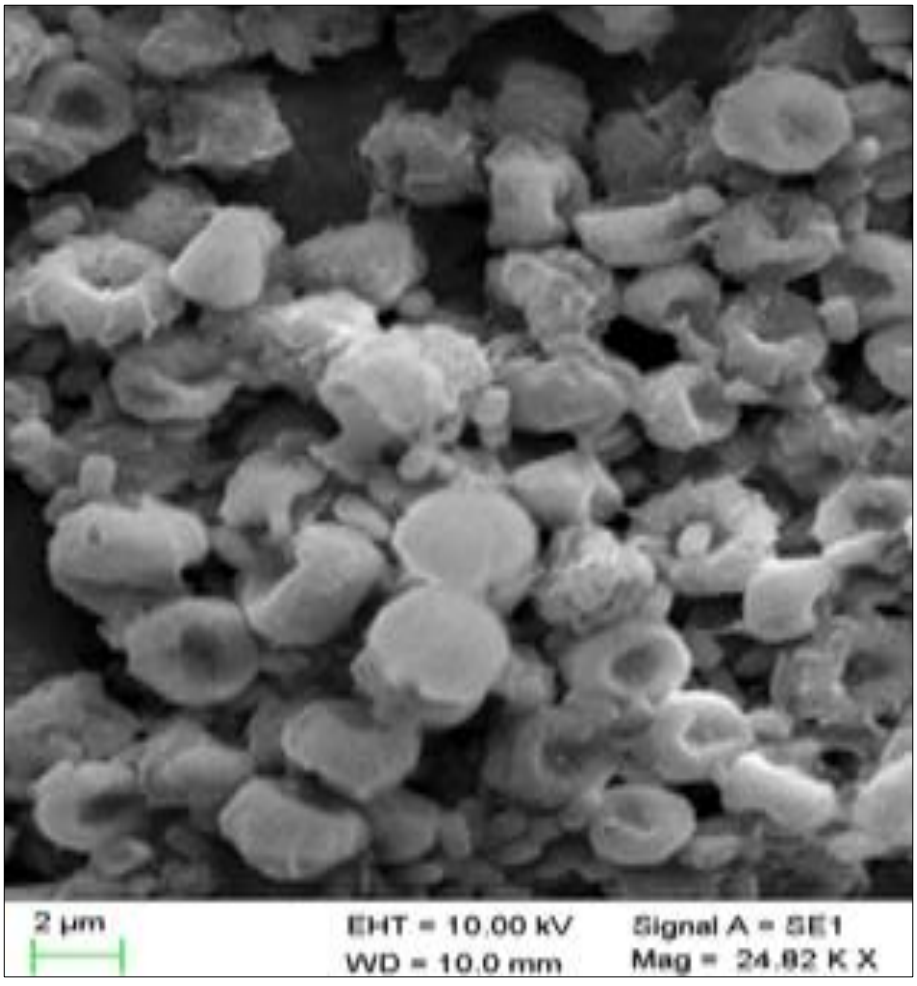

Fig 3: SEM

Sub-culturing of calli

The primary calli were cut into small pieces of approximately 4–5 mm size and transferred on the medium which was fortified with 2,4-D and Kn (2.0 + 0.5mg/L) for callus induction. Media was also supplemented with various doses of ABA and polyethylene glycol (control, 2, 6, 10 mg/L and control, 1.0, 1.5, 2 mg/L) respectively (Figure 4).

Another media syngertically supplemented with Polyethylene glycol, ABA and copper nanoparticles doses (control, 1.0 + 2 + 5 mg/L; 1.5 + 6 + 10 mg/L; 2 + 10 + 20 mg/L) respectively (Figure 5).

As a result, best regeneration of callus was observed after 45 days on media supplemented with 2, 4-D + Kn + AB + PEG (2.5 + 0.5 + 6 + 1.5mg/L) and 2, 4-D + Kn + ABA + CuNp + PEG (2.5 + 0.5 + 6 + 10 + 1.5 mg/L) (Figure 4b and 5b). The weight of callus was 1.04cm, 1.06gm and 1.15 cm, 2.04 gm respectively calculated.

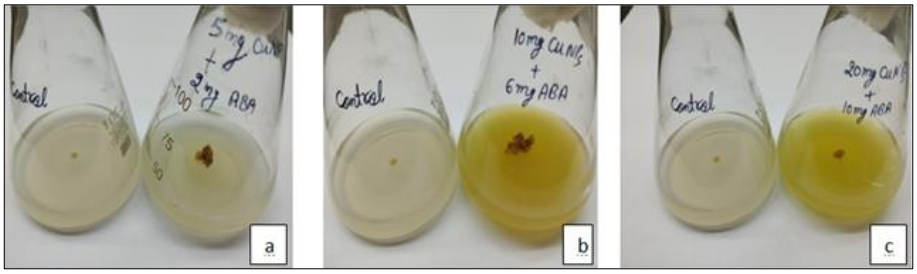

Fig 4: *In vitro* induction of calli of S. bicolor on MS media supplemented with different conc. of Cu-NPs and ABA (a) Control + Cu-NPS + ABA (5 + 2 mg/L) (b) Control + Cu-NPS + ABA (10 + 6 mg/L (c) Control + Cu-NPS + ABA (20 + 10 mg/L)

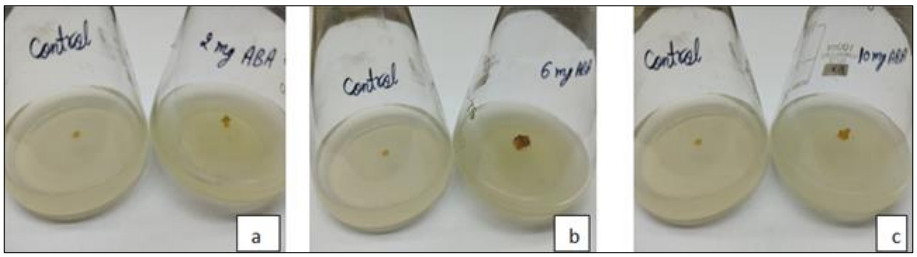

Fig 5: *In- vitro* induction of calli of S. bicolor on MS media supplemented with various conc. of ABA (a) Control + 2mg/LABA (b) Control + 6mg/L ABA (c) Control + 10mg/L ABA

Plant regeneration

Shoot induction

To induce shoots and proliferate shoots we cultured best regenerable calli (max. weight and max. proline and glycine betain content) on induction media and media fortified with different conc. and combination of BAP + Kn + IAA + IBA + NAA. The medium with higher conc. of BAP + Kn (2.0 + 0.3) MS_3; BAP + NAA (2.0 + 0.2) MS_6; BAP + Kn + NAA (2.1 + 1.0 + 0.3) S_{13} achieved the maximum and multiple shoot regeneration as shown in Table 7 and Figure 6.

Root induction

Regenerated shoots were transferred onto rooting medium for root development when their heights reached over 3cm. The media fortified with different conc. and combination of IAA + IBA + NAA. The medium with IBA + NAA (3.5 + 0.4) MS_{22} showed multiple roots regeneration (Table 7 and Figure 6)

Fig 6: Multiple shooting and rooting

Hardening and acclimatization

For hardening and acclimatization, fully formed plantlets with roots and shoots (10 cm in height) were transferred. During acclimatisation and hardening, every plantlet survived without suffering any damage (Figure 7).

Fig 7: Hardening and Acclimation

Quantification and estimation of proline and quantification of glycine betaine from *in vitro* culture

Glycine betaine (GB) and proline are the two most important organic osmolytes that accumulate in a variety of plant species in retort to environmental stresses such as extreme temperatures, drought, salinity, UV radiation and heavy metals. Lot of research and articles proved a positive connection between accretion of glycine betaine and proline and plant stress tolerance.

Free Proline content

The production of proline ranges from 1.3 to 3.4 mg/g FW on ABA application in drought condition as shown in Figure 8. Max. level of proline found 3.4mg/g FW as shown in Table 8 Proline range 1.8-2.7mg/g observed in calli on application of Cu-NPs and ABA dose syngertically. Max. proline observed was 2.7 mg/g Fw shown in Table 9. Regenerated plant from callus with (3.4 mg/g and 2.7 mg/g FW) showing the obvious tolerance under drought stress.

Table 7: Result and analysis for plant regeneration using different combinations of PGRs concentration

Growth Hormone	Modified MS media	Conc.(mg/L)	Biomass
1. MS + BAP + Kn	MS_1	1.0 + 0.1	C +
	MS_2	1.5 + 0.2	C + +
	MS_3	2.0 + 0.3	C + msh + + +
	MS_4	2.0 + 1.0	C + msh +
2. MS + BAP + NAA	MS_5	1.0 + 0.2	C + msh +
	MS_6	2.0 + 0.2	C + msh + + +
3. MS + Kn + IAA	MS_7	1.0 + 0.5	C + msh
	MS_8	1.0 + 0.5	-
	MS_9	2.0 + 1.0	C + msh +
	MS_{10}	3.0 + 1.0	-
4. MS + BAP + Kn + NAA	MS_{11}	4.0 + 2.0	-
	MS_{12}	1.0 + 0.1 + 0.1	C + msh +
	MS_{13}	2.1 + 1.0 + 0.3	C + msh + + +
5. MS + IAA + IBA	MS_{14}	2.0 + 0.3 + 0.3	C + msh +
	MS_{15}	1.0 + 1.0	C + R +
	MS_{16}	1.0 + 2.0	C + R +
	MS_{17}	0.5 + 3.0	C + R + +
	MS_{18}	2.0 + 5.0	C + R +
6. MS + IBA + NAA	MS_{19}	1.0 + 2.0	C + R +
	MS_{20}	2.0 + 3.0	C + msh +
	MS_{21}	3.0 + 0.5	C + R +
	MS_{22}	3.5 + 0.4	C + R + + +
	MS_{23}	3.0 + 0.4	C + R + +
S + /S + + /S + + + =Shoots produced, msh + /msh + + /msh + + + = Multiple shoot, R + /R + + /R + + + = Root produced Abbreviation			

Glycine Betaine

The production of glycine betaine ranges from 1.4 to 2.8mg/g on ABA application in drought conditions as shown in Figure 8. Max. level of glycine betain found 2.8mg/g as shown in Table 8. Glycine betaine range 1.6-2.1mg/g observed in calli on application of CuNPs and ABA dose syngertically Figure 9. Max. glycine betaine observed was 2.1mg/g as shown in Table 9. Regenerated plant from callus with (2.8mg/g and 2.1mg//L) showing the obvious tolerance under drought stress Table 8.

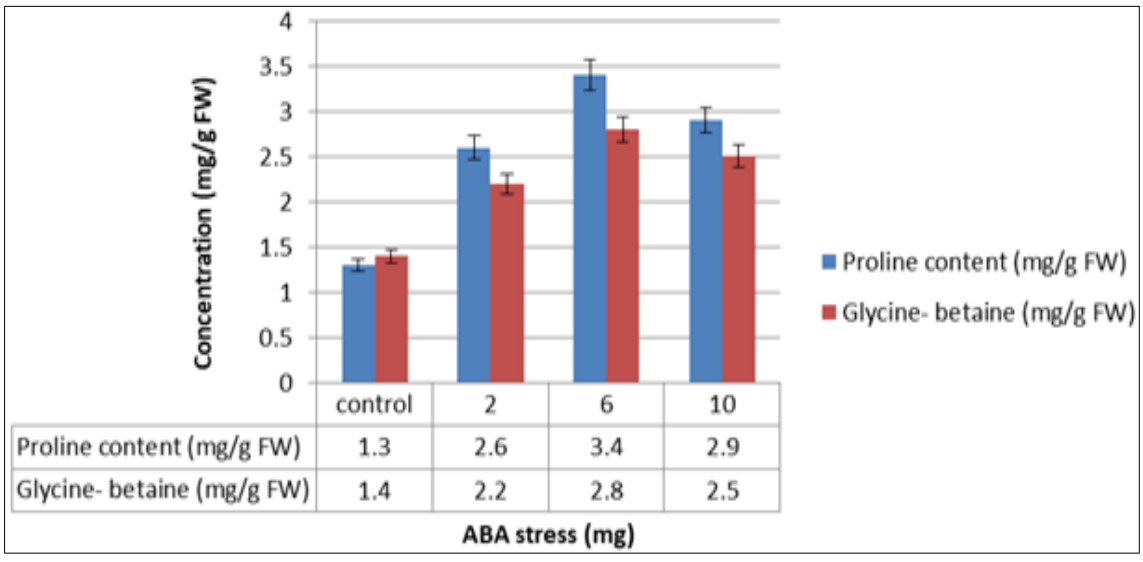

Fig 8: Proline and Glycine betaine content obtained from different conc. of ABA in callus media.

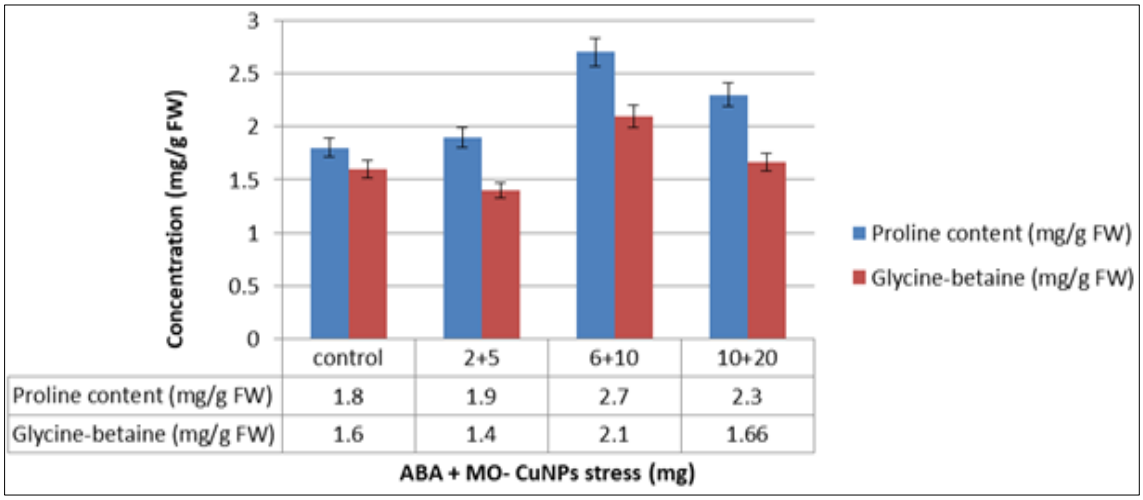

Fig 9: Proline and Glycine betaine obtained from syngertically supplemented different doses of Cu-NPS and ABA in callus media.

Table 8: Total proline and glycine betaine content obtained sing spectrophotometer (at λ=520 & 365 nm)

S. No.	ABA (mg/L)	PEG (mg/L)	2, 4D + Kn (mg/L)	Proline (mg/g)	Glycine betaine (mg/g)
1.	control	-	2.5 + 0.5	1.3±0.049	1.4±0.049
2.	2	1.0	2.5 + 0.5	2.6±0.05	2.2±0.05
3.	6	1.5	2.5 + 0.5	3.4±0.057	2.8±0.057
4.	10	2	2.5 + 0.5	2.9±0.052	2.5±0.052

Table 9: Total proline and glycine betaine content obtained using spectrophotometer (at λ=520 & 365 nm)

S. No.	ABA (mgL-1) + Cu-NPs	PEG (mg/L)	2,4D + Kn (mg/L)	Proline (mg/g)	Glycine betaine (mg/g)
1.	Control	-	2.5 + 0.5	1.8±0.049	1.6±0.049
2.	2 + 5	1.0	2.5 + 0.5	1.9±0.05	1.4±0.05
3.	6 + 10	1.5	2.5 + 0.5	2.7±0.057	2.1±0.057
4.	10 + 20	2	2.5 + 0.5	2.3±0.052	1.66±0.052

Discussion

In the present study we used seed as an explant. Explants made from seeds are thus unquestionably more practical and appropriate [11, 12, 13]. Thus the seeds were cultured on MS media supplemented with various growth regulators. Best callus induction obtained on the combination of 2, 4-D and kn at conc. of 2.5 + 0.5mgL^{-1} and it selected as the standard maintenance medium for subculturing totipotent calli. Similar study was reported by [20] 2, 4-D supplied @ 2.5 mg/L + kinetin, 0.5mg/l shows the best callus induction in rice seed. The study of [21] also showed the efficacy of plant growth regulators 2, 4-D and Kinetin in stimulating the production of callus at concentrations (2.5 and 0.9 mg.L-1). But [22] observed on half-MS medium with 2, 4-D and without kinetin, Maris Huntsman (wheat cultivar) formed a visible callus within 4 to 7 days of culture.

The positive effect of ABA have reported in previous study for drought tolerance and callus induction. In the present investigation different conc. of ABA is used. Max. callus growth and drought calli observed on 6mg/L and on increment on ABA conc. results in lowering the calls growth and drought tolerance. Assessment of abscisic acid effects have been limited in tissue culture even though it had became evident that this hormone induces *in vitro* some oxidases such as catalase (CAT). The study of [23] demonstrated that NO functioned as downstream of H_2O_2 to mediate and ABA-induced SOD (Superoxide dismutase) and CAT (catalase) activity.

The positive effects of metal-based NPs have been previously examined for drought tolerance in several other plant species. In present study, different conc. of Cu-NPs is used and max. callus growth and drought tolerant calli observed on 6 mg + 10mg/L (ABA + CuNPs) and increment in conc. of ABA and CuNPs results in lower callus growth and drought tolerant. Wheat productivity improved under water deficit conditions with Cu and Fe NP application through the increase in superoxide dismutase (SOD) activity and sugar content [24].

Glycine betaine (GB) and proline are the two most important organic osmolytes that accumulate in a variety of plant species in retort to environmental stresses such as extreme temperatures, drought, salinity, UV radiation and heavy metals. A positive connection between accretion of glycine betaine and proline and plant stress tolerance [25, 26]. Osmotic adjustment by proline defends cytosol from dehydration harm [27]. Among the functions of proline is that of maintaining the stability of structural proteins [29, 30] compatible osmolytes and antioxidant activity. The glycine betaine contents increased under abiotic stresses [31]. In present study, under control conditions the production of max. glycine betaine was 2.8mg/g on ABA application in drought condition. Max. Glycine betaine observed was 2.1mg/g on application of syngertic dose of ABA and CuNPS. Regenerated plant from callus with (2.8mg/g and 2.1mg/g) showing the obvious tolerance under drought stress. The production of proline max. Level of proline found 3.4mg/g FW on ABA application and max. proline 2.7mg/g Fw reported on application of CuNPs and ABA dose syngertically. Regenerated plant from callus with (3.4mg/g and 2.7mg/g FW) showing the obvious tolerance under drought stress.

Conclusion

The present result indicate that excellent callus resulted on

MS containing 2.5 + 0.5 mg/L (2, 4-D and Kintein). Resulted callus cultured on different doses of ABA and syngertic dose of ABA and CuNps. Positive effects were more considerable at its moderate dose ABA + PEG (6 + 1.5 mg/L); Cu-NPs + ABA + PEG (10mg/L + 6mg/L + 1.5mg/L). Higher dose demonstrated toxicity by negative impacts on accumulation of proline and glycine betaine. The positive response of sorghum to ABA and ABA + CuNPs under drought stress conditions cause to consider that ABA and CuNps + ABA as potential novel plant growth promoting and stress protecting agent.

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Conflict of interest
The authors do not have any conflict of interest.

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