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Titanium dioxide -mediated regulation of enzymatic and non-enzymatic antioxidants, pigments, and diosgenin content promotes cold stress tolerance in *Trigonella foenum-graecum* L.

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Abiotic stresses, notably cold stress, significantly influence various aspects of plant development and reproduction. Various approaches have been proposed to counteract the adverse impacts of cold stress on plant productivity. The unique properties of nanoparticles contribute to an enhanced tolerance of plants to challenging conditions. This study explores the impact of titanium dioxide nanoparticles (TiO_2 NPs) on cold-stress tolerance in fenugreek, as well as genes expression involved in the diosgenin biosynthesis pathway. Varied concentrations of TiO_2 NPs (0, 2, 5, and 10 ppm) were sprayed on fenugreek plants subjected to cold stress at 10 °C during 6, 24, and 48 h. Our findings revealed that the utilization of 2 and 5 ppm of TiO_2 NPs, positively influenced pigments biosynthesis and enzymatic and non-enzymatic antioxidant activities. It also effectively reduced electrolyte leakage and malondialdehyde content, mitigating the adverse effects of cold stress. The study also highlighted TiO_2 NPs' affirmative impact on defense signaling pathways, including abscisic acid, nitric oxide, and auxin, in fenugreek. Moreover, TiO_2 NPs significantly influenced the expression of genes related to diosgenin biosynthesis. Simultaneous exposure to cold stress and TiO_2 NPs led to a substantial increase in diosgenin content, with the upregulation of *SEP*, *SQS*, *CAS*, and *SSR* genes compared to control conditions. This research indicated that TiO_2 NPs application could effectively stimulate fenugreek biosynthesis of primary and secondary metabolites, consequently enhancing plant tolerance to cold stress. The study's outcomes hold promise for potential applications in the metabolic engineering of diosgenin in fenugreek.

Keywords Cold stress, Diosgenin, Fenugreek, Functional stimulator, Physiological changes, Titanium dioxide

The escalating global population and unfavorable environmental conditions leading to reduced agricultural production have sparked concerns worldwide. The adverse impact of environmental stresses, particularly on plant growth and yield, underscores the need for in-depth investigations into plant responses to these challenges[1]. Abiotic stresses, notably cold stress (CS), significantly influence various aspects of plant development and reproduction[2]. These stresses induce changes in plants' morphological, physiological, biochemical, and molecular characteristics, ultimately leading to adaptations to stressful conditions. CS induces molecular changes in plants, varying with the intensity and duration of exposure. In the short term, cold triggers membrane rigidification,

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increased reactive oxygen species (ROS), and activation of cold-responsive (COR) genes via transcription factors like CBF/DREB, leading to the production of protective proteins, osmolytes, and antioxidants. Medium-term exposure enhances these responses, with COR proteins stabilizing cellular structures, membranes undergoing lipid desaturation to maintain fluidity, and metabolic shifts prioritizing survival over growth. Prolonged cold or freezing leads to epigenetic modifications, increased accumulation of cryoprotective metabolites, and structural adaptations such as thicker cell walls and extracellular ice nucleation to prevent intracellular freezing. With persistent exposure, plants may enter dormancy to minimize metabolic activity. However, in cold-sensitive species, extreme or extended cold can overwhelm these defenses, causing irreversible cellular damage. These mechanisms illustrate plants' dynamic strategies for cold acclimation and freezing tolerance[2–4].

Various approaches have been proposed to counteract the adverse impacts of CS on plant productivity. One effective method involves the use of nanoparticles (NPs) to alleviate the harmful consequences of abiotic stresses[5]. Titanium dioxide nanoparticles (TiO_2 NPs) have garnered significant attention and found widespread use across industries. Numerous studies substantiate the positive effects of TiO_2 NPs in enhancing plant tolerance to abiotic stresses, providing compelling evidence of their efficacy[5,6]. A notable example demonstrating the efficacy of TiO_2 NPs treatment in mitigating the impact of cold stress can be observed in chickpea plants, resulting in distinct gene expression patterns related to cell signaling, cell defense, chromatin modification, and transcription regulation[4]. Furthermore, significant improvements were observed in photosynthesis efficiency, energy metabolism, and hydrogen peroxide (H_2O_2) content of chickpea plants, attributed to the positive regulation of genes involved in the biosynthesis of phosphoenolpyruvate carboxylase (PEPC), both subunits of RUBISCO, and chlorophyll a/b binding proteins upon the application of TiO_2 NPs. Notably, TiO_2 NPs stand out among other particles due to their high chemical resistance, non-toxicity, long lifespan, widespread availability, and cost-effectiveness[7]. Recent research has highlighted the significant role of TiO_2 NPs in effectively reducing the accumulation of malondialdehyde (MDA) through the induction of antioxidant systems[6]. Earlier studies have also attested that TiO_2 NPs can prevent oxidative stress and decrease membrane damage under CS conditions in plants[8].

Trigonella foenum-graecum (fenugreek), an annual plant of the Fabaceae family, is native to India and North Africa. With a rich history in culinary use and a common presence in herbal medicine, fenugreek stands out for its precious source of diosgenin[9]. Diosgenin, a biologically active steroid sapogenin, proves highly effective in treating various ailments, including hyperlipidemia, diabetes, cardiovascular disease, cancer, oxidative stress, and inflammation[10]. The application of stimulants and elicitors represents a highly successful strategy for enhancing the production of secondary metabolites, particularly those produced at low concentrations or absent under normal conditions[11,14]. Stimulants and elicitors play a crucial role in augmenting the biosynthesis of secondary metabolites by directly and indirectly inducing the expression of genes associated with their production[15].

The selection of the ideal planting date emerges as a pivotal factor in achieving maximum crop yield[16]. In Iran, fenugreek offers flexibility in planting, allowing for sowing in either autumn or spring. Autumn crops generally yield higher due to the extended growing season. However, delays in autumn planting may expose the plant to damage from CS[17]. To mitigate this challenge, the development of cold-tolerance varieties or the application of elicitors could be effective. The current study endeavors to evaluate the effectiveness of TiO_2 NPs in enhancing fenugreek plant tolerance to CS and to examine their impact on diosgenin content. Additionally, the study seeks to identify the optimal concentration of TiO_2 NPs for maximizing diosgenin production in fenugreek.

Results

Pigments content

Exposure to cold stress for 48 h resulted in a significant reduction in chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid contents, with decreases of 61%, 72%, 67%, and 58%, respectively ($p < 0.01$), compared to normal conditions without the application of TiO_2 NPs. However, when different levels of TiO_2 NPs were applied in the absence of cold stress, particularly at 2 and 5 ppm, there was a notable increase in chlorophylls and carotenoid contents compared to normal conditions (Table 1). The highest contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid were recorded at 8.50, 3.34, 11.84, and 3.43 mg g^{-1} FW, respectively, after 48 h of using 5 ppm TiO_2 NPs without cold stress. Interestingly, the application of different levels of TiO_2 NPs combined with cold stress not only prevented the reduction of chlorophyll and carotenoid contents but also led to their increase compared to cold treatment without TiO_2 NPs application. The results demonstrated that treating fenugreek plants subjected to 48 h of cold stress with 5 ppm TiO_2 NPs effectively boosted chlorophyll a (by 145%), chlorophyll b (by 200%), total chlorophyll (by 165%), and carotenoid (by 105%) contents when compared to cold stress alone (Table 1).

Electrolyte leakage and MDA content

The percentage of EC and MDA content exhibited a significant increase (2-fold, $p < 0.01$) with the prolongation of cold stress duration from 6 to 48 h, compared to normal temperature conditions. However, the treatment of plants subjected to cold stress, as well as plants at normal temperature, with different levels of TiO_2 NPs (especially 2 and 5 ppm) resulted in a significant reduction in EC percentage and MDA content. Interestingly, the highest values for these two indicators (MDA: 27 μmol g^{-1} FW and EC: 36%) were observed in the cold stress group treated with 10 ppm TiO_2 NPs, while the lowest values (MDA: 8.67 μmol g^{-1} FW and EC: 11%) were recorded with the application of 5 ppm TiO_2 NPs (Table 1). This suggests that TiO_2 NPs, particularly at lower concentrations, effectively mitigate the adverse effects of cold stress on cell membrane integrity and lipid peroxidation in fenugreek plants.

Soluble protein content and antioxidant activities

Cold stress, particularly after 48 h, resulted in a significant increase in soluble protein content ($p < 0.01$) compared to normal conditions (Table 2). Interestingly, the application of 5 ppm TiO_2 NPs, 24 and 48 h after application

Treatments		Chlorophyll a (mg g⁻¹ FW)	Chlorophyll b (mg g⁻¹ FW)	Total Chlorophyll (mg g⁻¹ FW)	Carotenoid (mg g⁻¹ FW)	Electrolyte leakage (%)	Malondialdehyde (μmol g⁻¹ FW)
Titanium Dioxide	Harvesting times						
23°C							
0	6 h	6.00 ± 0.01 fg	2.28 ± 0.07 f	8.28 ± 0.07 f	2.50 ± 0.10 f	14.11 ± 0.19 jk	10.50 ± 0.50 gh
	24 h	6.00 ± 0.01 fg	2.28 ± 0.07 f	8.28 ± 0.07 f	2.50 ± 0.10 f	14.11 ± 0.19 jk	10.50 ± 0.50 gh
	48 h	6.00 ± 0.01 fg	2.28 ± 0.07 f	8.28 ± 0.07 f	2.50 ± 0.10 f	14.11 ± 0.19 jk	10.50 ± 0.50 gh
2 ppm	6 h	6.018 ± 0.12 ef	2.54 ± 0.02 e	8.56 ± 0.11 e	2.60 ± 0.01 ef	13.87 ± 0.23 jkl	10.36 ± 0.34 gh
	24 h	6.45 ± 0.05 de	2.70 ± 0.09 d	9.15 ± 0.10 d	2.69 ± 0.02 de	13.20 ± 0.35 klm	10.10 ± 0.17 ghi
	48 h	6.75 ± 0.04 cd	2.85 ± 0.06 c	9.60 ± 0.08 c	2.87 ± 0.05 c	12.53 ± 0.50 mn	9.70 ± 0.26 hij
5 ppm	6 h	7.20 ± 0.06 b	2.76 ± 0.07 cd	9.87 ± 0.10 c	2.84 ± 0.06 c	12.93 ± 0.12 lmn	9.67 ± 0.58 hij
	24 h	8.22 ± 0.06 a	3.03 ± 0.06 b	11.25 ± 0.11 b	3.10 ± 0.10 b	12.67 ± 0.58 mn	9.10 ± 0.17 ij
	48 h	8.50 ± 0.14 a	3.34 ± 0.08 a	11.84 ± 0.17 a	3.43 ± 0.06 a	12.00 ± 1.00 no	8.67 ± 0.58 j
10 ppm	6 h	5.80 ± 0.06 gh	2.57 ± 0.03 e	8.37 ± 0.09 e	2.60 ± 0.05 ef	13.89 ± 0.34 jkl	10.19 ± 0.20 gh
	24 h	5.22 ± 0.04 i	1.80 ± 0.03 i	7.02 ± 0.07 j	1.68 ± 0.09 j	15.33 ± 0.58 hi	11.15 ± 0.26 g
	48 h	4.71 ± 0.06 j	1.24 ± 0.10 k	5.95 ± 0.09 l	1.19 ± 0.05 l	16.00 ± 1.00 gh	12.33 ± 0.58 f
10°C							
0	6 h	5.52 ± 0.06 hi	2.08 ± 0.07 g	7.60 ± 0.11 hi	2.12 ± 0.02 h	16.67 ± 0.58 g	12.33 ± 0.58 f
	24 h	3.78 ± 0.05 k	1.08 ± 0.08 l	4.86 ± 0.06 m	1.50 ± 0.07 k	23.67 ± 0.58 e	17.00 ± 1.00 d
	48 h	2.34 ± 0.04 m	0.63 ± 0.06 n	2.97 ± 0.02 o	1.05 ± 0.09 m	33.78 ± 0.69 b	24.00 ± 1.00 b
2 ppm	6 h	5.8 ± 0.06 gh	2.17 ± 0.08 g	7.97 ± 0.13 g	2.28 ± 0.08 g	15.49 ± 0.76 hi	10.67 ± 0.58 gh
	24 h	4.80 ± 0.03 j	1.63 ± 0.06 j	6.43 ± 0.09 k	1.96 ± 0.08 i	21.00 ± 1.00 f	14.00 ± 1.00 e
	48 h	3.54 ± 0.03 k	1.13 ± 0.06 l	4.67 ± 0.03 m	1.49 ± 0.06 k	30.33 ± 0.58 c	18.87 ± 0.81 c
5 ppm	6 h	6.33 ± 0.10 e	2.33 ± 0.06 f	8.66 ± 0.15 e	2.50 ± 0.03 f	11.00 ± 1.00 o	8.67 ± 0.58 j
	24 h	7.00 ± 0.06 bc	2.47 ± 0.06 e	9.47 ± 0.10 c	2.79 ± 0.11 cd	14.67 ± 0.58 ij	9.67 ± 0.58 hij
	48 h	5.76 ± 0.11 gh	1.94 ± 0.07 h	7.70 ± 0.13 i	1.92 ± 0.12 i	23.00 ± 1.00 e	13.67 ± 0.58 e
10 ppm	6 h	5.70 ± 0.10 gh	2.13 ± 0.06 g	7.84 ± 0.06 gh	2.30 ± 0.06 g	15.00 ± 1.00 hij	11.00 ± 1.00 g
	24 h	3.12 ± 0.06 l	0.86 ± 0.06 m	3.98 ± 0.11 n	1.15 ± 0.08 lm	25.00 ± 1.00 d	19.00 ± 1.00 c
	48 h	1.93 ± 0.03 n	0.53 ± 0.06 n	2.46 ± 0.03 p	0.88 ± 0.03 n	36.00 ± 1.00 a	27.00 ± 1.00 a

Table 1. The mean comparison effects of TiO₂ NPs concentrations and different harvesting times at 23 °C and 10 °C on some of the traits studied in this research. (The same letters represent insignificant differences at 1% probability level.)

Treatments		Catalase (nmol of H₂O₂ decomposed min⁻¹ mg⁻¹ protein)	Superoxide dismutase (nmol/min⁻¹ mg⁻¹ protein)	Ascorbate peroxidase (nmol oxidized ascorbate min⁻¹ mg⁻¹ protein)	Guaiacol peroxidase (nmol guaiacol min⁻¹ mg⁻¹ protein)	Soluble protein (mg ml⁻¹ protein)
Titanium Dioxide	Harvesting times					
23 °C						
0	6 h	17.49 ± 1.15 lm	14.46 ± 0.98 k	9.93 ± 0.06 k	15.33 ± 0.58 kl	64.33 ± 4.04 ij
	24 h	17.49 ± 1.15 lm	14.46 ± 0.98 k	9.93 ± 0.06 k	15.33 ± 0.58 kl	64.33 ± 4.04 ij
	48 h	17.49 ± 1.15 lm	14.46 ± 0.98 k	9.93 ± 0.06 k	15.33 ± 0.58 kl	64.33 ± 4.04 ij
2 ppm	6 h	19.00 ± 1.00 kl	15.33 ± 0.58 jk	10.33 ± 0.58 jk	15.67 ± 0.58 k	67.33 ± 0.58 i
	24 h	21.67 ± 0.58 hi	17.00 ± 1.00 hij	11.33 ± 0.58 ij	16.33 ± 0.58 ijk	72.67 ± 2.31 h
	48 h	25.33 ± 0.58 ef	20.00 ± 1.00 fg	12.33 ± 0.58 hi	17.67 ± 0.58 hi	82.33 ± 2.08 ef
5 ppm	6 h	23.33 ± 1.15 gh	18.67 ± 1.15 gh	12.67 ± 0.58 gh	17.67 ± 1.15 hi	78.33 ± 0.58 fg
	24 h	29.00 ± 1.00 d	25.00 ± 1.00 e	17.67 ± 1.15 f.	22.00 ± 1.00 f.	85.33 ± 2.52 e
	48 h	36.00 ± 1.00 c	31.00 ± 1.00 c	21.33 ± 0.58 e	25.33 ± 0.58 e	93.33 ± 2.89 d
10 ppm	6 h	21.00 ± 1.00 ij	16.00 ± 1.00 jk	11.33 ± 1.15 ij	16.00 ± 1.00 jk	68.33 ± 2.08 i
	24 h	14.33 ± 1.15 n	11.67 ± 1.15 lm	7.33 ± 0.58 l	13.67 ± 0.58 m	59.67 ± 0.58 k
	48 h	10.00 ± 1.00 p	8.67 ± 0.58 n	5.20 ± 0.72 m	11.33 ± 1.53 n	48.33 ± 1.53 l
10 °C						
0	6 h	19.33 ± 1.15 jk	16.67 ± 1.15 ij	12.00 ± 1.00 hi	17.33 ± 1.15 ij	68.33 ± 2.89 i
	24 h	25.67 ± 1.53 e	21.00 ± 1.00 f.	18.00 ± 1.00 f.	23.00 ± 1.00 f.	81.00 ± 1.73 f.
	48 h	34.67 ± 1.53 c	31.00 ± 1.00 c	25.67 ± 0.58 d	31.00 ± 1.00 c	95.67 ± 2.08 d
2 ppm	6 h	23.67 ± 0.58 fg	20.00 ± 1.00 fg	16.72 ± 1.11 f.	20.00 ± 1.00 g	75.33 ± 0.58 gh
	24 h	29.33 ± 1.15 d	24.13 ± 1.03 e	22.00 ± 1.00 e	28.33 ± 0.58 d	94.33 ± 4.04 d
	48 h	38.33 ± 1.53 b	29.00 ± 1.00 d	29.00 ± 1.00 c	36.00 ± 1.00 b	103.67 ± 3.21 c
5 ppm	6 h	28.33 ± 1.53 d	27.67 ± 0.58 d	22.33 ± 0.58 e	25.33 ± 0.58 e	94.33 ± 3.06 d
	24 h	38.67 ± 1.15 b	33.00 ± 1.00 b	31.67 ± 0.58 b	36.00 ± 1.00 b	108.33 ± 2.89 b
	48 h	49.00 ± 1.00 a	43.00 ± 1.00 a	41.00 ± 1.00 a	43.00 ± 1.00 a	118.33 ± 2.89 a
10 ppm	6 h	21.67 ± 0.58 hi	18.00 ± 1.00 hi	13.67 ± 0.58 g	19.00 ± 1.00 gh	75.33 ± 3.06 gh
	24 h	16.00 ± 1.00 mn	12.67 ± 2.08 l	9.67 ± 0.58 k	14.00 ± 1.00 lm	62.33 ± 1.15 jk
	48 h	12.00 ± 1.00 o	10.00 ± 1.00 mn	7.33 ± 0.58 l	10.00 ± 1.00 n	50.00 ± 2.00 l

Table 2. The mean comparison effects of TiO₂ NPs concentrations and different harvesting times at 23 °C and 10 °C on some of the traits studied in this research.

under non-stress conditions, further enhanced the soluble protein content by 33% and 48%, respectively, compared to normal conditions. Additionally, subjecting the plants to cold stress along with TiO₂ NPs resulted in a significant elevation in soluble protein content compared to cold stress treatment alone (without TiO₂ NPs application). The highest soluble protein content (118.33 mg g⁻¹ FW protein) was observed after 48 h of cold stress and 5 ppm TiO₂ NPs treatment, representing an 85% increase compared to the control (Table 2).

The activity of APX exhibited a notable increase upon lowering the temperature from 23 °C to 10 °C ($p < 0.01$). While extending the sampling time from 6 to 48 h and applying 2 and 5 ppm TiO₂ NPs resulted in

a significant elevation in APX activity, the application of 10 ppm TiO$_2$ NPs nearly halved the activity of this enzyme. Additionally, the highest recorded APX activity (41 nmol oxidized ascorbate min^{-1} mg^{-1} protein) was observed during 48 h of cold stress with 5 ppm TiO$_2$ NPs (Table 2).

In comparison to normal temperature, the CAT activity exhibited a sharp increase in response to applied cold stress. The utilization of 2 and 5 ppm of TiO$_2$ NPs significantly influenced this enzyme, with the activity doubling (100%) 48 h after the application of 5 ppm TiO$_2$ NPs compared to the control (without TiO$_2$ NPs and normal temperature). The simultaneous application of various levels of TiO$_2$ NPs and cold stress significantly heightened the activity of this enzyme. The highest CAT activity was recorded at 49 nmol of H$_2$O$_2$ decomposed min^{-1} mg^{-1} protein with the application of 5 ppm TiO$_2$ NPs and after 48 h of cold stress. However, applying 10 ppm TiO$_2$ NPs had an adverse effect on CAT activity and lowered it to 10 and 12 nmol of H$_2$O$_2$ decomposed min^{-1} mg^{-1} protein at 23 °C and 10 °C, respectively (Table 2).

GPX activity in plants subjected to cold stress was twice as high as in plants at normal temperature, particularly after 48 h of cold stress. Moreover, the application of 2 and 5 ppm of TiO$_2$ NPs in both temperature groups elevated the activity of this enzyme, with the highest GPX activity (43 nmol guaiacol min^{-1} mg^{-1} protein) observed under the application of 5 ppm TiO$_2$ NPs after 48 h of cold stress (Table 2). It is noteworthy that the application of 10 ppm TiO$_2$ NPs under both normal and cold conditions reduced the activity of this enzyme by 35% and 53%, respectively, compared to the control (without TiO$_2$ NPs application).

The application of cold stress significantly enhanced the activity of the SOD enzyme compared to the normal conditions ($p < 0.01$). Also, with the application of TiO$_2$ NPs and increasing the sampling time, the activity of this enzyme increased remarkably, with the highest amount observed in pots treated with 5 ppm of TiO$_2$ NPs after 48 h (43 nmol min^{-1} mg^{-1} protein). The concomitant application of 5 ppm TiO$_2$ NPs and 48 h of cold treatment enhanced SOD activity by 40% compared to cold stress alone (without TiO$_2$ NPs). At the same time, the effect of 10 ppm TiO$_2$ NPs and increasing the sampling time reduced the activity of this enzyme to some extent (Table 2).

Total phenol and proline content

Total phenol and proline content exhibited a significant increase ($p < 0.01$) with prolonged exposure to cold stress compared to the control. Moreover, using TiO$_2$ NPs without cold stress elevated these two traits compared to normal conditions. The application of 5 ppm TiO$_2$ NPs after 48 h under control temperature increased the total phenol and proline content by 67% and 90%, respectively, compared to the TiO$_2$ NPs-free condition. The application of various levels of TiO$_2$ NPs in cold-treated plants resulted in an elevation of these traits compared to cold stress conditions alone. Exposure to 48 h of cold stress along with the application of 5 ppm TiO$_2$ NPs raised the total phenol and proline contents by 200 and 205%, respectively, compared to the TiO$_2$ NPs-free and no cold-stress conditions. However, these compounds significantly decreased with the increase of TiO$_2$ NPs to 10 ppm (Fig. 1).

Phytohormones content

The impact of various durations (from 6 to 48 h) of cold stress on auxin content revealed a significant decline ($p < 0.01$) compared to normal conditions (Fig. 1). Under cold stress alone, auxin content decreased by 32% and 51% after 24 and 48 h, respectively, compared to normal conditions. The combined application of TiO$_2$ NPs and cold stress significantly enhanced auxin content compared to cold treatment alone. The highest auxin content, reaching 48.26 ng g^{-1} FW, was observed with the application of 5 ppm TiO$_2$ NPs after 48 h of cold stress (Fig. 1).

Moreover, cold stress led to a time-dependent increase ($p < 0.01$) in the ABA content compared to normal conditions. Cold stress raised ABA content by 60% after 48 h compared to the normal temperature. Additionally, the application of TiO$_2$ NPs without cold stress increased ABA content compared to control conditions. The
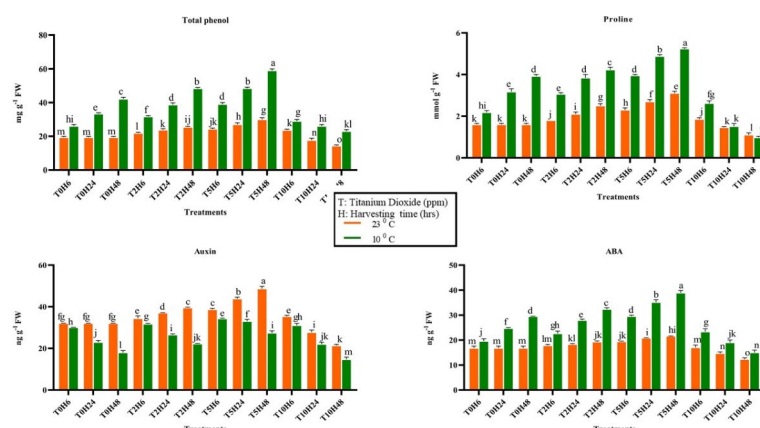

Fig. 1. The mean comparison of the effects of TiO$_2$ NPs concentrations and different harvesting times at 23 °C and 10 °C on the content of total phenol, proline, auxin, and abscisic acid. T$_0$, T$_2$, T$_5$, and T$_{10}$ indicate the levels of 0, 2, 5, and 10 ppm of TiO$_2$ NPs, respectively. H6, H24, and H48 represent different harvest times (6, 24, and 48 h) after the applied treatments, respectively. The mean values are compared using Duncan's method at a 1% probability level. The same letters represent insignificant differences.

simultaneous application of different levels of TiO_2 NPs and cold stress, especially 5 ppm after 48 h, caused a significant 37% increase in ABA content compared to cold stress treatment alone (without TiO_2 NPs application) (Fig. 1).

H_2O_2 content

The application of cold stress induced a significant increase ($p < 0.01$) in the H_2O_2 content compared to normal temperature conditions (Fig. 2). Specifically, after 24 and 48 h of cold stress, the H_2O_2 content increased by more than 2 and 3 times, respectively, compared to the control conditions. Interestingly, the addition of different levels of TiO_2 NPs to the pots experiencing 48 h of cold stress had a mitigating effect on the H_2O_2 content. In comparison to conditions without $TiO2$ NPs, the addition of TiO_2 NPs led to a reduction in H_2O_2 content by 24%, 75%, and 21%, respectively (Fig. 2).

NO content

The NO content in plants subjected to cold stress exhibited significant growth ($p < 0.01$), especially 48 h after cold stress, compared to normal conditions (Fig. 2). The application of various levels of TiO_2 NPs, particularly at 5 ppm under both normal and cold treatments, led to a notable increase in this trait compared to normal conditions (without TiO_2 NPs application). The simultaneous application of different levels of TiO_2 NPs and cold stress significantly elevated NO levels. However, the highest level of NO was recorded after 48 h of using 5 ppm TiO_2 (38 nmol g^{-1} FW) under cold stress (Fig. 2).

Gene expression analysis

The expression level of the *SQS* gene under normal temperature, with the application of 2, 5, and 10 ppm of TiO_2 NPs (24 h), increased by 4.7, 4.4, and 0.8-fold, respectively, compared to the control conditions (non-TiO_2 NPs) (Fig. 3). Treating plants exposed to cold stress with TiO_2 NPs significantly induced the expression of the *SQS* gene (in some treatments, not necessarily in all of them). Notably, the highest expression level of the *SQS* gene, reaching 32-fold over the control (normal temperature and without TiO_2 NPs application), was recorded when applying 24 h cold stress with the addition of 5 ppm of TiO_2 NPs (Fig. 3).

The results of this study indicated that the expression pattern of the *SEP* gene is significantly affected by the interaction between TiO_2 NPs concentrations and temperature (Fig. 3). Applying 2 ppm TiO_2 NPs under normal temperature and 48 h after sampling resulted in around a 13-fold increase in the expression of this gene compared to the control (without TiO_2 NPs). At normal temperature, by using 5 and 10 ppm TiO_2 NPs (24 h), the expression of this gene increased by 6 and 11 times, respectively, compared to normal conditions (without the application of TiO_2 NPs). The application of 2 ppm TiO_2 NPs at 6, 24, and 48 h after cold stress resulted in a 4, 7, and 8-fold increase in the expression pattern of the *SEP* gene compared to normal temperature (without TiO_2 NPs application) (Fig. 3).

The *CAS* gene expression considerably fell at 23 °C following the application of 2 and 5 ppm TiO_2 NPs compared to normal temperature without TiO_2 NPs application, while at 10 ppm, the expression level of *CAS* rose after 6 and 24 h, 20 and 8 times, respectively, compared to the control (Fig. 3). The expression of the *CAS* gene under cold stress without the application of TiO_2 NPs changes insignificantly compared to normal temperature and the non-application of TiO_2 NPs. The combined application of 6 h cold stress and 10 ppm TiO_2 NPs enhanced the expression of the *CAS* gene 15-fold compared to the control (23 °C and non-TiO_2 NPs application) (Fig. 3).

The study's findings unveiled a general decrease in the expression of the *SMT* gene in TiO_2 NPs-treated samples, both under normal and cold stress conditions (Fig. 3). Particularly noteworthy was the highest expression of the *SMT* gene observed when applying 2 ppm of TiO_2 NPs under normal temperature, resulting in a 1.4-fold increase compared to the control. However, the results indicated that exposing fenugreek plants to a combination of cold stress and TiO_2 NPs detrimentally affected the expression of the *SMT* gene, leading to a reduction in its value compared to the control at 23 °C without TiO_2 NPs application (Fig. 3).

Additionally, the study's findings suggest that the expression pattern of the *SSR* gene can be significantly influenced by the interaction of TiO_2 NPs concentrations and temperature treatments (Fig. 4). The application of 5 ppm of TiO_2 NPs (6, 24, and 48 h) under normal conditions increased the expression of the *SSR* gene by 3.5,
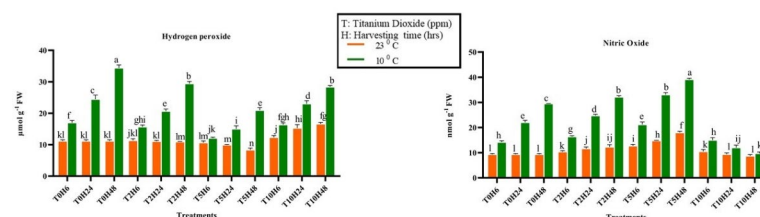

Fig. 2. The comparison effects of TiO_2 NPs concentrations and different harvesting times at 23 °C and 10 °C on hydrogen peroxide and nitric oxide content. T_0, T_2, T_5, and T_{10} indicate different concentrations of TiO_2 NPs (0, 2, 5, and 10 ppm, respectively) .H6, H24, and H48 represent different harvest times after the applied treatments (6, 24, and 48 h, respectively).The mean values are compared using Duncan's method at a 1% probability level. The same letters represent insignificant differences.
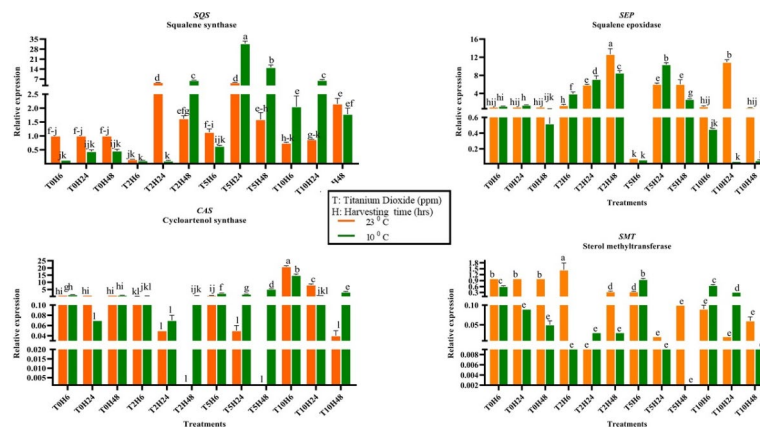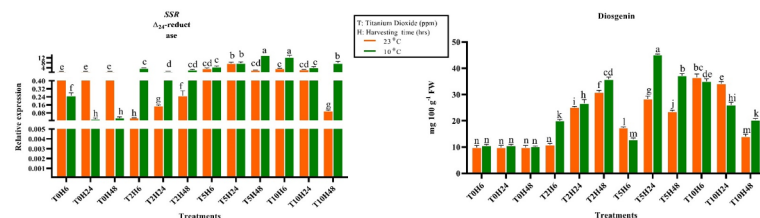
Fig. 3. The mean comparison of the effects of TiO$_2$ NPs concentrations and different harvesting times at 23 °C and 10 °C on the gene expression levels of *SQS*, *SEP*, *CAS*, and *SMT*. T$_0$, T$_2$, T$_5$, and T$_{10}$ indicate the levels of 0, 2, 5, and 10 ppm of TiO$_2$ NPs, respectively. H6, H24, and H48 represent different harvest times (6, 24, and 48 h) after the applied treatments, respectively. The mean values are compared using Duncan's method at a 1% probability level. The same letters represent insignificant differences.

Fig. 4. The mean comparison effects of TiO$_2$ NPs concentrations and different harvesting times at 23 °C and 10 °C on the *SSR* gene expression, and diosgenin content. T$_0$, T$_2$, T$_5$, and T$_{10}$ indicate the levels of 0, 2, 5, and 10 ppm of TiO$_2$ NPs, respectively. H6, H24, and H48 represent different harvest times (6, 24, and 48 h) after the applied treatments, respectively. The mean values are compared using Duncan's method at a 1% probability level. The same letters represent insignificant differences.

7.5, and 2 times, respectively, compared to the control. Under cold stress, the application of 5 ppm TiO$_2$ NPs (6, 24, and 48 h) resulted in a 4.7, 7.7, and 14-fold increase, respectively, compared to the control (Fig. 4).

Diosgenin content

Diosgenin content of samples was positively influenced by the application of various levels of TiO$_2$ NPs under normal temperature conditions, resulting in an approximately 2 to 3 times increase on average compared to the control (without the application of TiO$_2$ NPs) (Fig. 4). Furthermore, the combined application of cold stress and TiO$_2$ NPs significantly enhanced the diosgenin content in fenugreek compared to normal conditions. Specifically, subjecting plants treated with concentrations of 2, 5, and 10 ppm of TiO$_2$ NPs to cold stress, especially for 24 h, led to a sharp increase in diosgenin content by 195%, 430%, and 180%, respectively, compared to the control (no cold stress and no application of TiO$_2$ NPs). The highest recorded amount of diosgenin (90.33 mg g^{-1} FW) was observed in the treatment involving 24 h of cold stress and the application of 5 ppm of TiO$_2$ NPs (Fig. 4).

Discussion

In the present study, a decrease in chlorophylls and carotenoid content was observed under cold stress conditions (Table 1). This decline in chlorophyll biosynthesis might be attributed to the shared pathways of chlorophyll and proline biosynthesis, particularly through the common precursor glutamate. The increased accumulation of proline under cold stress could potentially disrupt chlorophyll biosynthesis, leading to a reduction in chlorophyll content[18]. Interestingly, in plants treated with TiO$_2$ NPs under cold stress, there was a significant increase in chlorophylls and carotenoid content. TiO$_2$ NPs, by diminishing the accumulation of H$_2$O$_2$ in fenugreek plants, appears to prevent the degradation of chlorophylls and carotenoids and stimulates their biosynthesis, especially at levels of 2 and 5 ppm after 6 and 24 h. The application of TiO$_2$ NPs has been reported to enhance the activity of various proteins involved in photosynthesis, including fructose 1–6 bisphosphatase in the Calvin cycle, gluconeogenesis enzymes, and enzymes related to the pentose phosphate cycle, which plays crucial roles in carbohydrate metabolism[19].

Under abiotic stress conditions, the production of ROS poses a risk to the stability of photosynthetic pigments. However, empirical evidence supports the efficacy of applying TiO$_2$ NPs in mitigating the negative

impacts of abiotic stresses. This mitigation is achieved by reducing H_2O_2 accumulation, thereby providing protection and enhancing the resilience of photosynthetic pigments against degradation[20]. Consistent with these findings, Parvin et al.[21] reported an increased abundance of photosynthetic pigments in *Linum usitatissimum* L. (Linaceae) when exposed to TiO_2 NPs. These results underscore the potential of TiO_2 NPs in safeguarding photosynthetic pigments and promoting their stability, even under stressful conditions.

Nanoparticles have the potential to influence various aspects of photosynthesis, impacting light absorption efficiency, electron transport, and carbon assimilation. They can alter both the structure and function of chloroplasts, affecting chlorophyll content, chloroplast membrane integrity, and the activity of photosynthetic enzymes. These modifications may lead to changes in photosynthetic rates, stomatal conductance, and assimilate generation, ultimately influencing plant growth and stress tolerance[22,24]. TiO_2 NPs, in particular, exhibit a dual role. Firstly, they enhance light energy absorption by regulating the expression of LHCII-b in the thylakoid membrane, facilitating its transfer from Photosystem I (PSI) to Photosystem II (PSII). Simultaneously, they expedite processes such as light energy conversion, electron transfer, photolysis of water, and oxygen evolution[25]. The application of NPs has been shown to augment the activity of the Rubisco enzyme by regulating the expression of miRNA51 and enhancing the activity of ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO)[23,26].

In general, NPs have the potential to mitigate the adverse effects of abiotic stresses by improving chlorophyll fluorescence parameters and amplifying Photosystem II (PSII) efficiency. NPs can interact with various components of plant cells, including cell membranes, organelles, and enzymes, influencing their structure and function. These interactions actively contribute to shaping crucial processes such as photosynthesis, respiration, water and nutrient absorption, and hormone signaling. These processes are essential for plants to effectively adapt to abiotic stresses[22,23,26].

In the present study, it was observed that under cold stress conditions, the MDA content and EC percentage significantly increased (Table 1). However, the application of different levels of TiO_2 NPs demonstrated a notable reduction in the percentage of EC and MDA content under cold stress conditions. This reduction indicates a decrease in the peroxidation of membrane fatty acids, suggesting that TiO_2 NPs can modulate the effects of cold stress on plant cells. While all concentrations of TiO_2 NPs effectively prevented the increase in these two indices, the concentration of 5 ppm proved to be particularly effective for both indices and significantly alleviated the negative effects of cold stress. The impact of TiO_2 NPs on reducing MDA content is highly dependent on the transmission of signaling pathways, ultimately regulating metabolic changes against oxidative stress in response to cold stress[27]. A related study suggested that low concentrations of TiO_2 NPs (5 ppm) during cold stress in chickpea genotypes resulted in a decrease in MDA content EC percentage[28].

Distinct and discernible patterns in H_2O_2 levels and antioxidant responses were observed in fenugreek plants subjected to various concentrations of TiO_2 NPs (2, 5, and 10 ppm) during different durations (6, 24, and 48 h) of cold stress. The results indicate an increase in total protein content under cold stress conditions, likely attributed to the upregulation of genes associated with primary metabolism, osmotic regulation, structural changes, as well as the increased expression of LEA (Late Embryogenesis Abundant) genes[29]. These genetic responses play a crucial role in adapting to and mitigating the effects of cold stress on fenugreek plants. Additionally, it was observed that the total protein content of fenugreek plants treated with 2 and 5 ppm TiO_2 NPs significantly increased under cold stress conditions. The augmentation in protein content can be attributed to the modulation of key genes regulating vital physiological and biochemical processes by TiO_2 NPs, consequently playing a role in enhancing plant metabolism[30].

Under cold stress conditions (48 h), the activity of antioxidant enzymes exhibited significant increases, including APX (160%), CAT (95%), GPX (100%), and SOD (105%), compared to normal conditions ($p < 0.01$) (Table 2). This study examined the activity of antioxidant enzymes, including CAT, SOD, GPX, and APX, which play an important role in clearing ROS and free radicals, and maintaining cell homeostasis and cell membrane stability. The results demonstrated that the application of TiO_2 NPs, particularly at concentrations of 2 and 5 ppm, significantly increased the activity of these enzymes in both temperature conditions (23 °C and 10 °C). The increased activity of these enzymes suggests that TiO_2 NPs have the potential to enhance the detoxification of H_2O_2 in plants by stabilizing cellular compounds and improving the physical properties of cell membranes[31]. This is in line with previous research conducted by Liu et al.[32], which revealed that the application of TiO_2 NPs under drought stress can enhance antioxidant enzyme activity, reduce lipid peroxidation, and improve membrane integrity in plants.

The activity of the antioxidant defense system in plants exposed to NPs is impacted by the augmentation of antioxidant gene expression, and biosynthesis of amino acids, nutrients, and osmolytes. Moreover, NPs prompt antioxidant activity in plants by significantly elevating the accumulation of fructose, glucose, trehalose, and sucrose[33]. Yang et al.[34] proposed that the enhancement of light absorption by TiO_2 NPs contributes to the improved conversion and transfer of energy from light, inhibiting chloroplast aging and ultimately enhancing the photosynthetic activity of chloroplasts. This could be attributed to the heightened activity of antioxidant enzymes like superoxide dismutase, peroxidase, and catalase, resulting from the intervention of TiO_2 NPs that serve to counteract excessive light exposure to the chloroplast. The utilization of TiO_2 NPs was noted to alleviate oxidative stress, diminish membrane damage, and enhance plant performance in cold conditions[35,36]. According to the findings of the present experiment, a concentration of 10 ppm of TiO_2 NPs exerted a detrimental impact on the activity of antioxidant enzymes (Table 2). Non-optimal concentrations of TiO_2 NPs could result in the deactivation of the antioxidant defense system, ultimately diminishing plant efficiency due to potential toxicity. Hence, it is imperative to pinpoint the optimal concentration of TiO_2 NPs for leveraging its role as a biosynthesis stimulator for primary and secondary metabolites, thereby allowing for the modulation of its impact on cold effects.

NPs have the capacity to interact with plant DNA, RNA, and proteins, resulting in modifications to gene expression and the activation of stress-responsive genes. These alterations can affect the synthesis of stress-

related proteins, osmolytes, and other molecules crucial for stress tolerance[23,37]. Another noteworthy outcome of the current study is the increased total phenol content observed under cold stress, particularly during the 48 h, and with the application of TiO$_2$ NPs, especially at 5 ppm, in comparison to the control. Additionally, the application of TiO$_2$ NPs (2 and 5 ppm) under normal temperature conditions led to a higher total phenol content than the control (Fig. 1). These results align with the findings of Karimizadeh et al.[38], who reported an increase in phenol accumulation in plants following the application of TiO$_2$ NPs. It is important to note that in response to oxidative stress, the activity of biosynthetic enzymes and the degradation of phenolic compounds change, leading to the accumulation of phenolic compounds in plants[6]. Numerous studies have documented the heightened activity of the enzyme phenylalanine ammonia-lyase (PAL), considered a catalyst for the phenol biosynthesis pathway[39,40]. In tobacco plants subjected to drought stress, the application of putrescine resulted in an increased activity of the PAL enzyme[41]. Phenols neutralize ROS by their capacity to donate oxygen atoms to free radicals[42,43].

According to our findings, the proline content significantly increased under cold stress, particularly after 48 h, compared to the normal conditions. Furthermore, various levels of TiO$_2$ NPs, including 2 and 5 ppm in each temperature group, elevated the proline content (Fig. 1). The amino acid proline accumulates extensively in plants in response to diverse stresses such as cold, salinity, and drought[44]. During stress conditions, proline collaborates with antioxidant enzymes to prevent the denaturation of cell membrane proteins, thereby safeguarding the structure and activity of proteins against damage caused by increased ROS and MDA production[45]. The utilization of NPs has been observed to enhance the proline content in plants, suggesting that these substances potentially enhance the activity of proteins and enzymes responsible for proline biosynthesis. As a result, they contribute to maintaining the photosynthetic cycle and regulating nutrient levels in plants[6]. In line with these findings, Mustafa et al.[46] reported that the exogenous application of TiO$_2$ NPs (40 mg/l) in two wheat varieties under salinity stress at concentrations of 100 and 150 mM acted as an osmotic protector against salt stress. This protective effect was demonstrated through the stabilization of cell membranes and preservation of enzyme integrity, ultimately leading to improved accumulation of soluble sugars and proline content in plants. The increase in proline accumulation in plants following TiO$_2$ NPs application is facilitated by the regulation of the *P5CS1* gene, which encodes d1-pyrroline-5-carboxylate. This gene plays a role in the synthesis of proline and sugar, contributing to the enhanced functionality of the cell membrane, proteins, and enzymes. Ultimately, this mechanism promotes the plant's tolerance under stress conditions[6,24,46].

Under normal conditions, ROS are not only produced in plants but also play a role in enhancing cell function by participating in cell signaling activities. However, when plants are exposed to biotic and abiotic stresses, the balance between ROS production and consumption is disrupted, leading to irreparable damage to the plant. Consequently, plants have evolved unique defense mechanisms to cope with stress[47]. The findings of this study indicated that cold stress treatment leads to an increased production of H$_2$O$_2$ as ROS. However, upon the application of different concentrations of TiO$_2$ NPs under normal temperature, the content of H$_2$O$_2$ gradually decreased, suggesting its potential role in mitigating the harmful effects of ROS. Moreover, treating stressed plants with 2 and 5 ppm of TiO$_2$ NPs effectively reduced the H$_2$O$_2$ content compared to cold treatment alone (Fig. 2). When plants face abiotic stresses such as cold and drought, TiO$_2$ NPs eliminate H$_2$O$_2$ by boosting the activity of antioxidants like CAT, SOD, and peroxidase[23]. In light of the current study's findings, the use of TiO$_2$ NPs could be considered a practical method to alleviate the adverse effects of cold stress. Our findings align with the research conducted by Weisany et al.[48] revealing that elevated H$_2$O$_2$ levels resulting from oxidative reactions in soybeans under salt stress lead to increased enzyme activity of CAT and APX. The heightened activity of antioxidant enzymes in plants treated with TiO$_2$ NPs under stress likely contributes to improved positive interactions and stronger signaling for the activation of additional defense mechanisms. The detoxification of H$_2$O$_2$ by NPs treatment may be linked to the stability of cell composition and the enhancement of the physical properties of the cell membrane[49–51].

In this study, plants exposed to 23°C and treated with 2 and 5 ppm of TiO$_2$ NPs exhibited minimal changes in H$_2$O$_2$ levels, while significant alterations in antioxidant enzyme activity were observed under these conditions. This outcome can be attributed to several factors[47].

1. Anticipation or preparation to cope with potential stresses: Plants may proactively increase the production of antioxidant enzymes in response to slight environmental changes, such as fluctuations in temperature, light, or nutrient levels, to be ready to cope with potential stressful conditions. In this case, enzymes rise to quickly break down ROS, such as hydrogen peroxide, if they increase[36,47].

2. Regulation of internal signaling: antioxidant enzymes, in addition to their role in scavenging ROS, can play a role in cellular signaling processes. The increase of these enzymes may be related to the internal settings of the plant and growth and development processes. For example, an increase in superoxide dismutase or glutathione peroxidase can lead to changes in signals regulating growth and differentiation[37,47].

3. Adaptation and development of defense mechanisms: Plants may enhance their defense mechanisms under the influence of mild environmental factors or even as part of the growth cycle. The increase of antioxidant enzymes may be a part of this adaptation process so that the plant can better cope with possible stresses such as water shortage, salinity, or light stresses in the future[37,47].

4. Controlling ROS levels at the signaling level: Under normal conditions, plants may want to keep ROS levels low enough to only act as signals to regulate biological processes, rather than reaching harmful levels. Increasing antioxidant enzymes can help maintain this delicate balance. In general, an increase in antioxidant enzymes without a change in H$_2$O$_2$ levels could indicate a preventive or regulatory mechanism in the plant that acts to protect or optimize physiological functions[36,47].

NO, serving as a crucial signaling molecule, plays a pivotal role in diverse biological functions, including seed germination, plant maturation and senescence, and the regulation of growth and development in plants[52,53]. The NO-dependent signaling pathway not only activates enzymatic and non-enzymatic defense systems but

also governs the production and elimination of ROS, lipid peroxidation, electrolyte leakage, and boosts the production of osmolytes such as proline[31,54]. Our research findings indicated that the application of cold stress, particularly after 48 h, and various concentrations of TiO$_2$ NPs, especially at 5 ppm, significantly increased NO (Fig. 2). Other studies have reported the positive effects of cold stress and TiO$_2$ NPs on increasing NO content[55]. In this study, the application of TiO$_2$ NPs led to an increase in NO content (Fig. 2). Additionally, numerous studies have indicated that elevated levels of NO and TiO$_2$ NPs can significantly enhance the activity of antioxidant enzymes[54–58]. The application of TiO$_2$ NPs in stressed plants, either by inducing the activity of the nitrate reductase enzyme and subsequently increasing NO content or by directly improving the activity of antioxidant enzymes, plays a role in promoting the stability of plant cells[54,56].

Nanoparticles possess the capability to influence the synthesis, metabolism, and signaling of plant hormones, including ABA, salicylic acid (SA), auxin, and jasmonic acid (JA). These hormones play crucial roles in regulating plant responses to various abiotic stresses such as drought, salinity, and heavy metal toxicity. Depending on their properties and concentrations, NPs can either enhance or suppress the production and signaling of these hormones[4,7,59].

In this study, cold stress resulted in a substantial reduction in auxin content, consistent with previous research indicating the impact of cold stress on the redirection of auxin signaling[60]. The application of various concentrations of TiO$_2$ NPs under normal temperature, especially at 5 ppm, led to an increase in auxin content (Fig. 1). Additionally, the auxin content under cold stress conditions, with the application of various concentrations of TiO$_2$ NPs, was significantly higher compared to cold stress conditions without the application of TiO$_2$ NPs. A recent study on soybeans also reported an elevation in auxin content following the application of TiO$_2$ NPs[61]. Several reports have highlighted the impact of TiO$_2$ NPs on plant growth regulators, specifically auxin, leading to increased root length and regulation of auxin in Arabidopsis[62,63]. The results of this study suggest that subjecting fenugreek plants to cold stress in the presence of TiO$_2$ NPs could potentially enhance auxin content by upregulating the expression of auxin biosynthesis genes.

The findings of this study also demonstrate a significant increase ($p < 0.01$) in ABA content under cold stress conditions and with the application of 2 and 5 ppm of TiO2 NPs, compared to the control group (Fig. 3). This observation is consistent with the results of other researchers[64,65]. The application of TiO$_2$ NPs at normal temperature also increased ABA content, aligning with the findings of Chaca et al.[66]. Additionally, Syu et al.[67] observed that the application of silver NPs in Arabidopsis elevated ABA levels by enhancing the expression of the gene encoding the rate-limiting biosynthetic enzyme NCED3. It is noteworthy that the promoter regions of several genes associated with heavy metal stress contain ABA-responsive elements (ABRE), suggesting a strong correlation between ABA content and the levels of gene transcripts[68]. The presence of NPs appears to stimulate cellular redox status and phytohormone production, leading to the generation of specific signaling pathways that impact the transcription of nuclear genes. Extensive research has been conducted on the effects of NPs on phytohormones, NO, and gene transcription in various plant species[69]. NPs can alter DNA methylation patterns, histone modifications, and small RNA expression, leading to changes in gene expression and stress tolerance. Investigating the epigenetic mechanisms underlying NPs-induced stress responses could provide a deeper understanding of their long-term effects on plant physiology and adaptation[70,71].

Notably, the combination of 24 h of cold stress and the application of 5 ppm TiO$_2$ exhibited the highest level of *SQS* gene expression, reaching 31.84 (Fig. 3). Our findings revealed that the application of 2 ppm of TiO$_2$ NPs at normal temperature after 48 h significantly increased the expression of *SEP* by 13 times compared to the control. When 2 ppm of TiO$_2$ NPs was applied after 6, 24, and 48 h of cold stress, there was a 4, 7, and 8-fold increase in the expression of this gene, respectively, compared to the control (Fig. 3). These findings suggest that the application of TiO$_2$ NPs as a functional elicitor may effectively enhance the expression of this gene. The expression pattern of the *CAS* gene differed from that of the two previously mentioned genes (*SQS* and *SEP*), indicating that under cold stress conditions without the application of TiO$_2$ NPs, the expression of *CAS* did not change significantly compared to the control. Moreover, the application of 10 ppm TiO$_2$ NPs after 6 h at both temperatures of 23 °C and 10 °C resulted in a 20-fold and 15-fold increase in the expression of this gene, respectively, compared to the control (at 23 °C and without titanium dioxide) (Fig. 3).

In our study, the expression of the *SMT* gene was reduced in NPs-treated samples under both normal and cold stress conditions. The highest expression of this gene was observed with the application of 2 ppm of TiO$_2$ NPs at normal temperature (1.4 times) (Fig. 3). The results indicated that exposure of fenugreek to cold stress and TiO$_2$ NPs had a negative impact on *SMT* gene expression, leading to a reduction in its expression compared to the control (at 23 °C without TiO$_2$ NPs). However, the expression patterns of the *SSR* gene revealed that the interaction between TiO$_2$ NPs and cold stress significantly influenced its expression (Fig. 4). For instance, with the application of cold stress (6, 24, and 48 h) along with 5 ppm of TiO$_2$ NPs, *SSR* gene expression increased by 4.7, 7.7, and 14-fold, respectively, compared to the control.

It appears that the *SSR* gene governs the diosgenin biosynthesis pathway, while the *SMT* gene intervenes in the process, directing the pathway toward the production of plant sterols and similar compounds[72,73]. Given that the expression of the *SMT* gene (a rival gene) remained almost unchanged, and the expression of the *SSR* gene (the main gene) increased with the application of cold stress and TiO$_2$ NPs treatment, it can be concluded that the simultaneous application of TiO$_2$ NPs and cold stress may be an efficient approach to enhance diosgenin content (Fig. 4). This aligns with findings in the thymoquinone biosynthesis pathway in *Nigella sativa*, where the expression of main genes increased with the induction of TiO$_2$ NPs[74]. Zhang et al.[75] proposed that silver NPs enhanced the production of secondary metabolites in *Artemisia annua*. With the application of these NPs, the weight of artemisinin increased from 1.67 to 2.86 mg per gram of dry weight[76]. The findings of Moshirian Farahi et al.[77] demonstrated that TiO$_2$ NPs played a crucial role in driving changes in the expression of genes in Vitex plants. The induction of this NP affected the plant's antioxidant enzymes and altered the expression of genes involved in the terpenoid biosynthesis pathway.

The small size of TiO_2 NPs allows them to efficiently and rapidly penetrate plant cells, thereby stimulating gene expression. TiO2 NPs can act as stressor factors, enhancing plant defense activities and thereby triggering the expression of genes responsible for secondary metabolite production in plants[78]. Karimzadeh et al.[38] documented that the foliar application of TiO_2 NPs under drought stress increased the expression of the RAS gene. Similarly, it has been noted that the utilization of silver NPs augments the expression of *tydc7*, *DBOX*, *DIOX2*, and *bbe1* genes in *Papaver somniferum* L[79].

The behavior of NPs can significantly differ based on their concentration and structure. Lower concentrations of NPs tend to stimulate the biosynthesis of both primary and secondary metabolites, while higher concentrations are more likely to induce toxicity and disrupt cellular processes[80]. The findings of the current study also affirmed that (for most traits, though not universally), the application of 2 and 5 ppm of TiO_2 NPs effectively mitigated the impact of cold stress on the plant and resulted in an increase in the expression of genes related to diosgenin biosynthesis. In contrast, the use of 10 ppm of TiO_2 NPs had fewer positive effects and, in some cases, even demonstrated negative effects. The outcomes of diverse studies underscore the impact of NPs on stimulating the biosynthesis of secondary metabolites. In alignment with our findings, it has been documented that selenium NPs are linked to the stimulation of secondary metabolism by inducing the expression of *RAS* and *HPPR* genes in Melissa officinalis[69]. Sheikhi et al.[14] reported that subjecting fenugreek plants to heat stress with 24-epibrassinolide (8 µM for 6 h) significantly upregulates the expression of *SQS*, *SEP*, *CAS*, and *SMT* genes, along with a notable increase in diosgenin content by 8, 2.8, 11, 17, and 6 times, respectively.

The rise in essential oil production observed with TiO_2 NPs application in plants is congruent with the enhancement of growth conditions, photosynthesis, the expression of genes involved in secondary metabolite biosynthesis, and defense mechanisms. The improvement in essential oils in TiO_2 NPs-treated plants can be attributed to increased access to substrates and essential components, along with specialized biosynthesis enzymes[23]. Our findings align with the outcomes of Ebadollahi et al.[81], who reported that TiO_2 NPs positively stimulates the synthesis of secondary metabolites in plants under stress conditions through diverse signaling cascades, including ROS, mitogen-activated protein kinases, calcium flux, gene expression, and the activation of specific metabolic enzymes. NPs elevate the expression of genes associated with the generation of hormonal signals, including GmWRKY27, GmMYB118, and GmMYB174, as well as the synthesis of secondary metabolites and defense mechanisms. Consequently, this influence contributes to the enhancement of plant tolerance under stress conditions[82,83].

NPs possess the capacity to interact with various components of signaling pathways in plants, including receptors, kinases, and transcription factors. These interactions can lead to the modulation of stress-related gene expression and the activation of downstream signaling cascades[7,84]. Understanding the molecular basis of NPs-mediated modulation of signaling pathways is crucial for elucidating the mechanisms underlying NPs-induced stress tolerance in plants. By deciphering the specific signaling events and molecular interactions involved, researchers can gain insights into the intricate network of stress-responsive genes and pathways that are influenced by NPs[7,84,86]. By manipulating these pathways, it may be possible to improve plant tolerance to various abiotic stresses, leading to increased crop productivity and sustainability. It is important to note that while the field application of TiO_2 NPs holds promise, further research is needed to fully understand their long-term effects, potential risks, and optimal application methods. Responsible application and regulation of nanotechnology in agriculture are crucial to ensure its safe and sustainable implementation. Overall, the application TiO_2 NPs in agriculture offers a range of mechanisms and potential benefits, including enhanced nutrient uptake, improved water use efficiency, seed germination, hormonal regulation, and environmental remediation. These NPs have the potential to revolutionize agricultural practices and contribute to more sustainable and productive farming systems[84,87].

Materials and methods
Plant materials and treatments preparation
The taxonomy of the studied plant was confirmed by a specialist botanist from the Ministry of Agriculture Jihad of Tehran, Iran. The plant material was obtained under the supervision and permission of the Ministry of Agriculture Jihad of Tehran, Iran as well as national guidelines, with all authors complying with all local and national guidelines. For this study, fenugreek seeds sourced from the Boshruyeh genotype were sterilized using a 2% sodium hypochlorite solution for 10 min. Following sterilization, the seeds underwent multiple rinses with sterile water before being planted in pots measuring $20 \times 25 \times 30$ cm. The potting mixture comprised equal parts of peat moss, perlite, and sand. The growth chamber maintained a consistent photoperiod of 16–8 h, with temperature settings between 23 and 25 °C and humidity levels ranging from 55 to 60%. Fluorescent lamps, with a light intensity of 400 µmol $m^{-2} s^{-1}$, were employed to ensure adequate illumination for the plants.

For the study, TiO_2 NPs with an average size of 20–30 nm, existing in the 99.7% anatase form, were procured from Sigma-Aldrich, USA. TiO_2 NP solutions were prepared using double-distilled water at concentrations of 2, 5, and 10 ppm, as established by previous studies[4,8,28]. These concentrations were chosen based on preliminary experiments. To ensure proper dissolution and achieve a nanoscale solution, ultrasonic treatment was applied using an Elmasonic P model ultrasonic bath for 45 min. Seedlings, measuring 15 cm in height and featuring 5–8 leaves (At 4 weeks old), were subjected to spraying with three different levels of TiO_2 NPs solution (2, 5, and 10 ppm). Each concentration of TiO_2 NPs was sprayed uniformly on all plants using an automatic sprayer, with approximately 10 cc applied per plant. The pots were randomly divided into three groups; the control group received a spray of double-distilled water. Subsequently, one group remained in standard conditions (23 °C), while the other was placed in a growth chamber set at 10 °C to induce CS. Sampling occurred at 6, 24, and 48 h after the initiation of treatments. Leaf samples were promptly frozen in liquid nitrogen and stored at -80 °C. The middle leaves from the apex of plants in each treatment were selected for property measurements, assuming a similar physiological age for all seedlings[4,8,28].

Pigments assay

To quantify chlorophyll (a, b, and total) and carotenoids, 500 mg of shredded leaves were homogenized in 5 ml of 80% acetone. After centrifugation at 3500 rpm for 15 min at 4 °C, the supernatants were carefully transferred to new Falcon tubes, and their volumes were adjusted to 10 ml using 80% acetone[88]. Finally, a spectrophotometer (UV-1800) was utilized to determine the light absorption at wavelengths of 480, 645, and 663 nm, providing accurate measurements of pigment concentrations. Chlorophyll and carotenoid contents were ultimately calculated using the following formulas:

Chlorophyll a: 11.75 (A663) – 2.35 (A645).

Chlorophyll b: 18.61 (A645) – 3.96 (A663).

Carotenoid: 1000 (A480) – 2.27 Chla– 81.4 Chlb/229.

Where A = Absorbance, Chla = chlorophyll a (mg/L), Chlb = chlorophyll b (mg/L), Carotenoids (mg/L). For converting the concentrations from mg/l to mg/g fresh weight, each value multiplied by (extraction volume/(sample weight*1000))[89].

Electrolyte leakage percentage

To prepare leaf samples for electrolyte leakage (EC) assessment, a punching machine was employed to create 1 cm diameter discs from intact leaves. These discs were subsequently placed in Falcon tubes containing 10 ml of distilled water, ensuring complete water absorption by the samples using a vacuum pump. The Falcon tubes were then positioned in a shaker, and the electrolytic conductivity (Ec1) of the samples was measured with an EC meter from Weilheim, Germany. To determine Ec2, the samples underwent immersion in a hot water bath for one hour. The damage index was calculated using the formula established by Hepburn et al.[90]:

% EL=(Ec1 / Ec2) * 100. This formula allowed for the accurate quantification of electrolyte leakage as a percentage.

Measurement of malondialdehyde content

The determination of malondialdehyde (MDA) content followed the method introduced by Heath and Packer[91]. In summary, 2 ml of extraction buffer (0.5% trichloroacetic acid) was homogenized with 250 mg of shredded leaf samples and centrifuged at 3500 rpm for 15 min. Subsequently, 1 ml of the resulting supernatant was combined with 2 ml of a 0.5% thiobarbituric acid solution containing trichloroacetic acid (20% TCA). The mixture was incubated in hot water (90 °C) for 20 min, followed by prompt transfer to an ice bath for the same duration. After centrifugation at 3500 rpm for 15 min, the samples were measured at 532 and 600 nm using a UV-1800 spectrophotometer. Using the following equation, the quantity of MDA was calculated[92]:

MDA = [(532 –600 nm)/(QD × QF)] × DF. In this formula, QD = Cuvette diameter (1 cm), QF = Extinction coefficient (155 mmol/cm), and DF = Dilution factor (20).

Assessment of total protein and antioxidant enzyme activity

To evaluate the total protein content and the activities of antioxidant enzymes, namely catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), and guaiacol peroxidase (GPX), an enzyme extract was prepared according to the following protocol. Initially, 0.5 g of ground leaf tissue underwent blending with 2 ml of phosphate buffer (50 mM, pH 7.0) and subsequent centrifugation at 3500 rpm for 15 min at 4 °C. The resulting supernatant was utilized for conducting the antioxidant enzyme activity assay. In accordance with widely accepted methods, the total protein content was determined following Bradford's protocol[92]. Simultaneously, the activities of CAT[93], APX[94], SOD[95], and GPX[96] were measured using a UV-1800 spectrophotometer.

Total phenol content measurement

1 gram of each dried plant sample was initially mixed with 10 ml of 80% methanol at 40 °C and subjected to shaking for 24 h. Following filtration with filter paper, the resulting extract was combined with 0.5 ml of Folin–Ciocalteu reagent and 4 ml of 1 M Na_2CO_3 solution. The mixture underwent incubation for 15 min at room temperature. Finally, the absorbance of the samples was measured at 765 nm using a UV-1800 spectrophotometer. For each sample, three replications were performed, and the standard curve of gallic acid (Merck, CAS Number: 149-91-7) at eight concentrations served as the reference to quantify the total phenol content[97].

Proline content assay

The proline content was assessed following the method developed by Bates et al.[98]. In this procedure, 100 mg of shredded fresh leaf tissue was thoroughly mixed with 10 ml of a 3% sulfosalicylic acid solution, and the resulting mixture was filtered through Whatman filter paper. Subsequently, an equal amount (2 ml) of the obtained extract, pure hydrogen reagent, and pure acetic acid were thoroughly mixed and subjected to a hot water bath at 90 °C for 60 min. The samples were promptly transferred to an ice bath for 10 min, and 4 ml of toluene was added to the solutions. The red phase, containing the amino acid proline, formed at the top. To determine the proline content, the absorbance of the samples was recorded at 520 nm using a UV-1800 spectrophotometer.

Determination of hydrogen peroxide content

The leaf tissue (0.5 g) was finely ground and thoroughly mixed with 5 ml of 0.1% trichloroacetic acid (TCA), immediately before being centrifuged at 35,000 rpm. Next, a mixture, composed of the supernatant (750 µl), 10 mM potassium phosphate buffer (750 µl), and 1 mM potassium iodide (1.5 mL) was accurately prepared and their absorption rate was recorded at 390 nm. It is worth noting that, owing to the sensitivity of H_2O_2, this experiment was conducted in relative darkness on ice (4 °C)[99]. Ultimately, the H_2O_2 content was measured using standard curves (Sigma, USA, CAS Number: 7722-84-1).

Gene	Forward	Reverse	Length product (bp)	Melting point (°C)
Sterol side chain reductase (*SSR*)	GGAGGCATCATACTTCTGT	CATCACTTCCTGGATTGTA	210	60
Δ24-reductase sterol methyltransferase (*SMT*)	ACGAACAAGTTCCAGGTC	GGTGACAGGGATACAAGAG	230	60
Squalene epoxidase (*SEP*)	GGGGTTATTCAGATGGGTC	CCACCAGCCATTACTACAC	220	61
Cycloartenol synthase (*CAS*)	CGAGGAGATTGGTGCTGGTG	TCGTGTTGGGTGGAGATAAG	230	60
Squalene synthase (*SQS*)	TTATCACCATTGGTGCTGAG	CGATGTTTCCATACAGATCCTT	160	61
Glyceraldehyde 3-phosphate dehydrogenase-Reference gene (*GAPDH*)	ATGTTAAATGATGCAGCCCTTCCACCTCTC	TATGTTTGTTGTTGGTGTCAACGAGCAACGAATACAAG	230	61

Table 3. The primers used in the current study. (The same letters represent insignificant differences at 1% probability level).

Nitric oxide content assay

To measure Nitric Oxide (NO) content, the methodology outlined by Zhou et al.[100] was followed. In this process, 3 ml of 50 mM cold acetic acid buffer containing 4% zinc acetate with a pH of 4 was added to 0.6 g of shredded leaf tissue. The samples underwent two-stage centrifugation: initially, centrifugation at 4 °C and 3000 rpm for 15 min, followed by washing the supernatants with 1 ml of extraction buffer and repeating the centrifugation under the same conditions. In the subsequent step, the resulting supernatant was thoroughly mixed with 0.1 g of activated charcoal and filtered using an appropriate filter. The filtered solution (1 ml) was then mixed with 1 ml of grease agent and left for 30 min at room temperature. The absorption rate of the solution was measured at 540 nm. To quantify the NO content, a $NaNO_2$ standard curve was employed (Sigma, USA, CAS Number: 7632-00-0).

Phytohormones content measurement

For the assessment of auxin content, an extract was prepared following the method described by Zhou et al.[101]. Subsequently, HPLC analysis (Agilent Technologies Inc., USA, 1200 series) was conducted, as per the methodology proposed by Cao et al.[102]. The auxin content in leaf tissue was determined using HPLC with a flow rate of 0.8 ml/min, employing a C18 column (4.6 μm, 250 mm length, and 5 mm diameter). The HPLC column was maintained at a temperature of 30 °C, and a gradient of 0.45% formic acid and acetonitrile was utilized. The gradient included the following steps: 0–5 min with a ratio of 95:5% (v/v) of formic acid/acetonitrile, 5–6 min transitioning from 95:5% to 0:100% (v/v), and finally, 6–16 min with a ratio of 0:100% (v/v). To determine auxin concentration, a linear regression equation from standard (Sigma, USA, CAS Number: 87-51-4) calibration curves was applied. The peak areas at the maximum wavelength of 260 nm were considered for quantification.

The evaluation of abscisic acid (ABA) content utilized the method introduced by Hubick and Reid[103]. An HPLC column (Agilent Technologies Inc., USA, 1200 series, C18, 4.2 μm, 250 mm length, and 5 mm diameter) was employed according to standard ABA. Chromatographic separation involved two mobile phases with water/acetonitrile/formic acid in volume ratios of 94.9:5:0.1 (A) and 10:89.9:0.1 (B). The elution program included maintaining 100% A for 5 min, followed by two consecutive linear gradients from 0 to 6% B in 10 min and from 6 to 100% B in 5 min, with a final maintenance of 100% B for another 5 min[104]. A flow rate of 0.8 ml/min was applied at a wavelength of 254 nm. Quantification was performed based on the specific area of the observed peak concerning the retention time of the standard abscisic acid sample, obtained from Sigma, USA (CAS Number: 21293-29-8).

RNA extraction and cDNA synthesis

RNA extraction was carried out using the RNeasy plant mini kit (Qiagen) following the manufacturer's instructions. To eliminate DNA contaminants, one microgram of the extracted RNA was treated with DNase (Thermo Fisher Company). Subsequently, the quality and quantity of RNA were assessed using a 1% agarose gel and a NanoDrop spectrophotometer. The RNA concentration for all samples was standardized to 200 ng/μL for cDNA synthesis. For cDNA synthesis, 20 μl of complementary DNA (cDNA) was synthesized using the Reverse Transcriptase kit (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions. Primers were designed using the Primer 3 Plus online program, and their accuracy was verified using Oligo Analyzer v.3.1 (http://eu.idtdna.com/calc/analyzer) (Table 3).

Real-time PCR reaction

For the assessment of relative gene expression via real-time PCR, SYBR®Green PCR Master Mix 2X (Ampliqon) was utilized. The real-time PCR reaction volume was 10 μl, and the experiment was three biological and technical replications. It is worth noting that the concentration of the synthesized cDNA was 1000 ng/μL, and the concentration of cDNA used in the real-time PCR reaction was adjusted to 100 ng/μL. The temperature and time conditions consisted of 35 cycles at 95 °C for 20 s and 60 °C for 40 s, followed by a single cycle at 95 °C for 15 min. To ensure the samples were uncontaminated, a negative control was included in the experiment.

Diosgenin content assay
The evaluation of diosgenin content followed the method introduced by Zolfaghari et al.[72]. Utilizing an HPLC column (Agilent Technologies Inc., USA, 1200 series, C18, 4.2 μm, 250 mm length, 5 mm diameter) at a flow

rate of 0.8 ml/min and a wavelength of 210 nm, diosgenin content was measured. The mobile phase comprised acetonitrile and water in a 90:10 ratio, with a flow rate of 0.7 ml and a wavelength of 210 nm[105]. Various concentrations of diosgenin (Sigma-Aldrich-Germany; CAS Number: 512049), including 100, 150, 300, 500, and 1000 ppm, were prepared by dissolving the compound in acetonitrile and subjecting it to ultra-sonication for 30 min.

Data analysis

The current research was structured as a factorial experiment based on a completely randomized design. Following the completion of an analysis of variance, the Duncan test was employed for mean comparisons using SPSS 26 software (IBM SPSS, Armonk, NY, USA). For the real-time PCR data analysis, the relative standard curve method was applied. The accuracy of the raw data was ensured by examining melting and amplification curves. The analysis followed the formula $2^{-\Delta\Delta Ct}$, as introduced by Livak and Schmittgen[106].

Conclusion

This investigation explored the use of TiO_2 NPs to enhance the plant immune system and bolster fenugreek's resilience to cold stress. The application of these NPs demonstrated several positive effects, including the prevention of a decline in chlorophyll and carotenoid content under cold stress and a significant increase in the levels of these valuable compounds. Additionally, TiO_2 NPs boosted the activity of antioxidant enzymes, creating favorable conditions for the breakdown of H_2O_2 following cold stress. This, in turn, prevented an escalation in fatty acid peroxidation and the subsequent leakage of these compounds from the cell. The study also examined the impact of TiO_2 NPs and cold stress on the expression of specific genes in the diosgenin biosynthesis pathway. The results suggested that TiO_2 NPs could modify the expression of fenugreek genes by activating signaling pathways. In summary, the findings indicate that TiO_2 NPs may serve as effective stimulants in the biosynthesis of both primary and secondary metabolites, particularly diosgenin. Moreover, they have the potential to mitigate the adverse effects of cold stress on fenugreek, highlighting their possible application in agriculture for enhancing plant resilience and productivity.

Data availability

The data generated or analyzed in this study are included in this article. Other materials that support the findings of this study are available from the corresponding author on reasonable request.

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MJB. Investigation and Methodology; AE. Conceptualization, Data curation, Formal analysis, Project administration, Supervision, Validation; Visualization, Writing-Original Draft, Writing -Review & Editing; PH. Supervision, Validation, Review & Editing; EA. Formal analysis and Review & Editing; ZCH. Formal analysis, Validation; Visualization and Review & Editing. SHGH. Formal analysis, Validation, and Review & Editing.

Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

This article does not involve any studies conducted on human participants or animals by any of the authors. Commercial, or advanced breeding genotypes were used in the study, and no wild material was used.

Additional information

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