Journal of Soil Science and Plant Nutrition (2025) 25:4112–4124
https://doi.org/10.1007/s42729-025-02387-y

ORIGINAL PAPER

Efficacy of Peroxidase Activity and Isozyme as Molecular Markers for Assessing Iron Deficiency and Toxicity Via in Vitro Culture as a Rapid Technique in Banana

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Received: 15 September 2024 / Accepted: 13 March 2025 / Published online: 8 April 2025

Abstract
In arid and semi-arid zones, iron (Fe) deficiency represents great challenge for banana cultivations. There is no available standard table for fertilization program of banana in these areas. Therefore, the current study aimed to test the in vitro culture technique as a more rapid methodology for screening the appropriate Fe level and its relation to the activity and isozymes fingerprints for peroxidase in "Williams" banana plantlets. After the fourth subculture of multiplication, the experiments were started. The tested concentrations of Fe relative to Fe content in the Standard Murashige and Skoog medium (MS) were 0%, 100%, 200%, 300%, 400%, 500%, 1000% and 1500%. The analogous concentrations of Fe were 0, 5.50, 11.0, 16.5, 22.0, 27.5, 55.0 and 82.5 mg L^{-1}, respectively, which applied in a completely randomized design using ten replicates. The obtain results proved that removing Fe from MS caused Fe deficiency and the most common symptom was chlorosis of the entire lamina, then all plantlets turned yellow or white. On the contrary, the excess concentration in Fe (82.5 mg L^{-1}) in MS tended to blacken the shoots and arrested growth. Removing Fe from the medium gave the lowest value of peroxidase activity. Contrariwise, peroxidase activity was progressively increased by elevating concentration of Fe in the medium. The activity of peroxidase remained stable in plantlets grown in 11, 16.5 and 22 mg L^{-1} Fe. Thereafter, a sharp increase in activity was observed in plantlets grown in 27.5 mg L^{-1} Fe. This increase continued to reach the maximum in plantlets grown in the medium supported with the highest Fe concentration (55 mg L^{-1}). It could be concluded that there is a positive relationship between Fe concentration in MS-medium and peroxidase isozymes. This is useful in diagnosing iron deficiency or toxicity in laboratory, affording the opportunity to perform various tests to obtain rapid information that can be used in constructing an accurate fertilization program schedule for banana under field conditions.

Keywords Banana plantlets · Fertilization scheduling · Molecular indicators · Nutrient phyto-toxicity · Tissue culture

Abbreviations

Fe	Iron
MDA	Malondialdehyde
MS	Murashige and Skoog medium
POD	Peroxidase
ROS	Reactive oxygen species
SPAD	Relative chlorophyll content

1 Introduction

Banana is one of the oldest fruit tree cultivated by man since the pre-historic times. It is grown widely in tropical and subtropical climates with highly profitable fruit yield (Zahra et al. 2021a). In recent years, banana cultivation is

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increasing in Egypt, and the expansion has included a large area of sandy soil (Ali et al. 2018). No doubt, in these poor soils, crops require special cultural practices specially fertilization either with macro- and micro- nutrients (Saudy and El-Metwally 2019; Rizk et al. 2023). Nutrients deficiency is a biotic stress that adversely influenced physiological status and nutrient balances, thus plant growth and development (Saudy 2014; Saudy et al. 2020a; Abdo et al. 2024). The hazard impacts of deficit nutrition extended to decline yield and quality (Saudy and Mubarak 2015; Saudy et al. 2020b; Hadid et al. 2024). Therefore, the artificial supply of mineral is a crucial practice in crop management for correcting the deficiency of nutrients (Noureldin et al. 2013; Abd–Elrahman et al. 2022; Lasheen et al. 2024). Fertilization programs of most fruits trees are based on leaf analysis of nutrients and these programs include the critical levels of any given nutrient to know the different ranges of each one (Rozane et al. 2016). These levels are of great importance in diagnosing the mineral status of the plant, which in turn will help determine the appropriate fertilization program for banana plants. In Egypt, as a remarkable producer of banana, such information is not available (Abd El-Latif et al. 2020).

Tissue culture technique was recently used as a new tool for studying the effect of different micronutrients on growth and mineral uptake in plantlets of various crops (Oberschelp and Gonçalves 2018). In tissue culture propagation, Murashige and Skoog (MS) medium is widely utilized as a nutritional growing medium. To study the deficit or surplus nutritional statuses of a specific nutrient, nutrient composition of MS medium could be altered (Radouani and Lauer 2015; Nguyen et al. 2021).

Since iron (Fe) participates in a large number of metabolic processes, it has been categorized as an essential nutrient element for growth and development of all living organisms involving plants (Connorton et al. 2017). Despite Fe exists in relatively significant amount in soils, its soluble form is low (Kim and Guerinot 2007; Ramadan et al. 2023, 2024a). In soils with a high chemically active calcium carbonate contents, i.e. calcareous soils, plants suffer from Fe deficiency, hence growth and yield decline (Dey et al. 2021; Salem et al. 2021; Saudy et al. 2023). Thus, in crop fertilization programs, supplying Fe becomes a crucial act to improve crop yield and quality (Saudy et al. 2021, 2022; Elgala et al. 2022). However, in banana cultivation in open field there is no standard nutritional programs, specifically for Fe to be applied in arid zones such as Egypt. To assess the appropriate level of Fe, preliminary studies should be adopted for examining the levels that could cause nutrient deficiency/toxicity in plants. In this concern, Muleo et al. (1995) carried out an experiment on quince clones "MA" and "Ct.S.212" to screen Fe limiting conditions in in vitro culture. Herein, six levels (concentrations) of Fe in MS medium were established. Fe in the MS medium was decreased gradually (100%= 0.10 mmol Fe) to (80%, 65%, 50%, 25% and 15% of that of standard content) in order to induce chlorosis symptoms. Amounts expressed in equivalents of Fe-EDTA were, 200, 160, 130, 100, 50 and 30, respectively. Results showed that "Ct.S.212" clone presented a higher proliferation rate and greater growth than clone "MA" in the 15 and 25% Fe treatments, but clear symptoms of chlorosis were observed in both clones at the two lowest Fe concentrations (25 and 15% of standard content). Several researchers have shown that not all iron absorbed by plants is transported and not all transported iron is absorbed and assimilated by leaf cells (Mubarak et al. 2021; Abd El-Mageed et al. 2022; Salem et al. 2022; Saudy et al. 2022). For these reasons total plant Fe composition is generally not only a good measure of the plant´s Fe nutritional status but also enzymatic methods offer another approach for assessing the mineral nutritional status of plants (Shaaban et al. 2023; Ali et al. 2024a). These assessments are based on the fact that the activity of certain enzymes such as peroxidase (POD) is lower or higher depending on the nutrient in deficient than in normal tissue (James 1984; Marschner 1995). They added that parameters of vegetative development commonly used in in vitro culture proved to be difficult to interpret for this type of screening, and further tests particularly mineral analysis will be necessary. In this respect, Al-Shabi (2002) studied the genetic diversity for the selected most tolerant and sensitive sorghum cultivars under Fe deficiency and salt stress using POD isozymes based on native polyacrylamide gel electrophoresis. The results indicated that the most sensitive and tolerant cultivars revealed four bands, which were not completely present in all cultivars. The most sensitive cultivars were characterized by the appearance of band 2 at migration distance of the protein (RF) of 0.13 comparing with tolerant cultivars. Whil, the tolerant cultivars were similar in their electrophoretic patterns. He added that POD isozymes were more efficient to study the genetic diversity for sensitive and tolerant cultivars for Fe deficiency and salt stress evaluated in the greenhouse. There is a strong relationship between Fe level and activity of peroxidase enzyme. In this regards, Fe is linked with enzyme biosynthesis associated with peroxidase pathways, which are directly included in cell growth, root development, catabolism of auxin and lignification (Lima et al. 2018). Further, Fe enhanced the antioxidant defense mode under stress via booting the activity of peroxidase (Moradbeygi et al. 2020).

Despite the availability of studies related to establishing the deficient or excess nutrient status in plants via tissue culture technique (Radouani and Lauer 2015; Munthali et al. 2022), no studies have been implemented for tissue culture to investigate the deficiency/excessive of Fe impacts on banana. In this work, it has been hypothesized that there is a

relationship between Fe level in growth medium of banana plantlet and molecular changes expressed in POD activity and isozymes. Therefore, the present work involved two main objectives: The first objective was to test the in vitro culture technique as a more rapid methodology for screening micronutrient levels especially Fe. The second one was to study the effect of Fe levels on nutrient contents and POD activity and isozymes fingerprints in "Williams" banana plantlets grown in vitro.

2 Materials and Methods

This study was carried out on in vitro banana shoots of "Williams Hybrid. The study was carried out in the Tissue Culture Laboratory of Strawberry and Non Traditional Crops Improvement Center., Faculty of Agriculture, Ain Shams University, Egypt. Plant materials (shoot meristems) were previously obtained from Laboratory of Tissue Culture, Horticulture Institute, Giza. Egypt.

2.1 Tissue Culture Procedures

In vitro clusters of "Williams" banana grown in ten jars each of 200 ml capacity from the second subculture of multiplication stage were kindly supplied from the Tissue Culture Laboratory. In order to produce a large number of in vitro banana shoots, the stock salt solution of Murashige & Skoog-1962 standard medium **(** MS **)** for tissue culture was prepared as shown in Table 1. Moreover, the medium required 30 g/l sucrose, 7.0 g/l agar and hormone (5.0 mg/l butyric acid). The pH was adjusted to 5.7–5.8 using NaOH and HCl. The medium was poured into 200 ml jars (85 × 50 mm). For each jar, 50 ml medium was added and then autoclaved at 100 K.Pa and 121°C for 20 min. Jars were left to cool and incubated at 25 ± 2°C for 3 days before transferring banana clusters.

Table 1 Murashige and Skoog-1962 Medium (MS) Components for Tissue Culture

Component	(mg/l)
NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_2O$	370
KH_2PO_4	170
$CaCl_2$	332
H_3BO_3	6.20
$MnSO_4.4H_2O$	16.9
$ZnSO_4.7H_2O$	8.60
$CuSO_4.5H_2O$	0.025
KI	0.830
Co $Cl_2.6H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.250
$FeSO_4.7H_2O$	27.50
Na_2EDTA	37.30
Vitamins	
Thiamine HCl	0.1
Pyridoxine HCl	0.5
Nicotinic acid	0.5
Glycine	2.0
MyoInositol	100

After the fourth subculture of multiplication, a large number of in vitro banana shoots was obtained and the experiments were started to study the effect of different concentrations of Fe, in MS medium on in vitro banana shoots. The standard medium was modified by application of Fe (ferrous sulfate, 19.7% Fe) based on Fe levels which were eight concentrations of Fe (0, 5.5, 11.0, 16.5, 22.0, 27.5, 55.0 and 82.5 mg L^{-1}) to obtain eight different media). The experimental treatments were arranged in a completely randomized design using ten replicates. Each replicate (jar) contained four shoots (1–1.5 cm in length) planted in 200 ml jar involving 50 ml solid MS medium supplemented with 3.0 mg/l 6-benzylaminopurine, benzyl adenine, (BA) and 1.0 mg/l indole butyric acid (IBA). Routine re-culturing was carried out every four weeks. Re-culturing was carried out for two times and in each re-culture the previous proliferated shoots were re-cultured on a fresh medium contained the same mentioned components. It also should be pointed out that all the previous cultures were incubated at 25 ± 2°C under photoperiod cycle of 16/ 8 h as light/ dark. Light intensity was adjusted at 3000 lx, using cool white fluorescent lamp.

2.2 Assessments

2.2.1 Morphological Characteristics

After the second re-culture (three months from the beginning the treatments application, the plantlets were removed from the different media, washed with distilled water to asses **s** relative chlorophyll content (SPAD values) in leaf blades (Süß et al. 2015), length of original shoots, plantlet fresh weight. After that, plantlets were oven dried at 60–70 °C until a constant weight to measure plantlet dry weight.

2.2.2 Chemical Analysis

Dried plantlets were ground by means of stainless steel rotary knife mill and each of nitrogen (N), phosphorus (P), potassium (K), Fe, zinc (Zn) and manganese (Mn) were determined (Cottenie et al. 1982). Each of N, P and K content were expressed as percent of dry matter, whereas Fe, Zn and Mn were calculated as parts per million (mg L^{-1}). Moreover, mineral uptake of plantlets was calculated depending on the plantlet dry weight.

2.2.3 Activity and Isozyme Fingerprints for Peroxidase

The activity and isozyme fingerprints for POD as a metabolic indicator for Fe content in plant tissues were estimated. In this respect, the plant material taken from the leaves of in vitro culture plants which were treated by the different concentrations of Fe. The main objective of this study was to test the POD activity in relation to the different levels of Fe (Marschner 1995). Also the isozyme fingerprints for POD was studied. To determine these measurements, the following steps were performed:

2.2.3.1 The Extraction of Peroxidase Enzyme Fresh leaves samples (blade and midrib) from banana plantlets about (1.0 g) was ground in 5 ml from 50 mM phosphate buffer (pH 6.4) in ice bath and then centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant (crude enzyme extract) was used to determine POD activity and POD isozymes.

2.2.3.2 Peroxidase Activity The POD activity was determined by using the guaiacol oxidation method (Hammerschmidt et al. 1982). In this method, the reaction mixture (3 ml) consisted of 100 µl from 0.25% (v/v) guaiacol, 2.7 ml from 10 mM sodium phosphate buffer (pH 6.0), 100 µl of the crude enzyme extract and 100 µl from 10 mM hydrogen peroxide were added to initiate the reaction which was measured spectrophotometrically at 470 nm. Total POD activity was determined by means of a spectrocolourimeter and expressed as the increase in absorbance at 470 nm min^{-1} g^{-1} fresh weight.

2.2.3.3 POD Isozymes Fingerprint Native polyacrylamide gel electrophoresis was used to study the isozymes content for POD (Stegeman et al. 1985). In this investigation the isozymes content for POD of banana was determined which was affected by different concentrations of some micronutrients Fe in MS medium. The stock solution buffer used for isozymes electrophoresis was prepared as follows:

Gel buffer solution (Tris Borate buffer pH 8.9): the stock solution was composed of 60.5 g Tris [(hydroxy methyl) aminomethane] and 46.0 g boric acid dissolved in 5 L distilled water. Electrode buffer (0.125 M pH8.9): was prepared by diluting 300 ml of the stock solution (Tris Borate buffer pH 8.9) with 2100 ml distilled water. Acrylamide stock 30%: was prepared by dissolving 29.2 g acrylamide and 0.8 g N, N methylene bis-acrylamide dissolved in 100 ml distilled water. Ammonium persulphate 2%: was prepared by dissolving 0.25 g ammonium persulphate in 10 ml distilled water. This stock must be prepared immediately before use.

2.2.3.4 The Preparation of Gel This was prepared by adding 35 ml of 30% acrylamide with 70 ml (0.125 M pH 8.9) electrode buffer to get 8% acrylamide, 2.5 ml ammonium persulphate and 0.66 ml TEMED (tetra methylene diamine) were added. The gel solution was quickly poured in 15 well combs, and then the gel was left about 30 min for polymerization.

2.2.3.5 Application of Samples A volume of 70 µl from the supernatant (crude enzyme extract) of each sample was mixed with 10 µl bromophenol blue and 10 µl glycerol. Then the mixture was loaded on the gel. The run was carried out at 100 V for about 2 h. The solution of reaction mixture which was used for pod visualization consisted of 0.25 g benzidine dihydrochlorid, 5 drops glacial acetic acid and 100 ml distilled water. Ten drops (about 0.5 ml) of freshly hydrogen peroxidase were added to the reaction mixture just before staining. The gel was shakered about one minute and incubated at room temperature until the bands appeared, then the reaction was stopped with tap water and the bands were appeared clearly then the gel was photographed. Estimation of isozymes bands was carried out by gel analyzer program version-3 (available free on the internet) at software: http://www.geocities.com/egy.gene.

2.3 Statistical Analysis

Each treatment was replicate six times on four-shoots/jar-plots in a completely randomized design and all data obtained were statistically analyzed by using the analysis of variance (casella (2008). Means were differentiated by duncan's multiple range test at 5% level of significance.

3 Results

3.1 Morphological Characteristics

The obtained results proved that length of original shoot (Fig. 1), plantlets fresh weight (Fig. 2) and plantlets dry weight (Fig. 3) were significantly affected by the level of Fe in MS medium. The highest significant values of the three traits were obtained by basic MS. Except for the concentration of 16.5 mg L^{-1} Fe, increasing Fe concentration in media higher than 5.5 mg L^{-1} showed significant reductions in length of original shoot. Plantlet fresh and dry weights significantly declined as Fe concentrations increased above 5.5 mg L^{-1}. The maximum greenness (SPAD) values were observed with 11.0, 5.5 and 16.5 mg L^{-1} Fe (Fig. 4). The maximum reductions in all morphological traits were

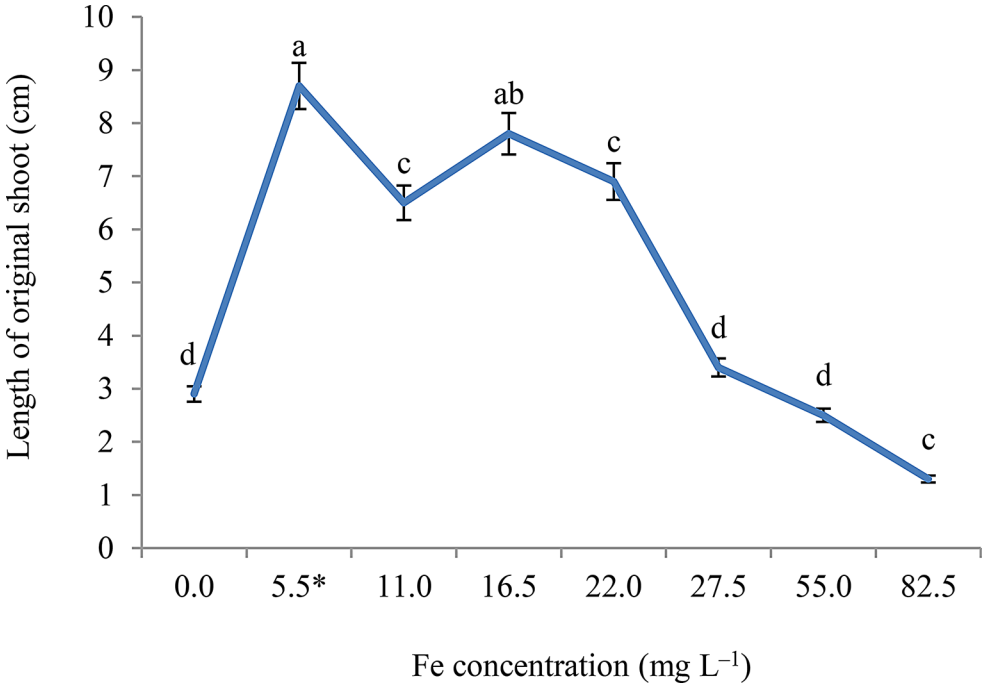

Fig. 1 Effect of different levels of iron (Fe) on length of original shoot of "Williams" banana plantlets grown in in vitro. *= Concentration of Fe in standard Murashige and Skoog medium (MS). Values with the same letter (s) are not statistically different according to Duncan's multiple range tests

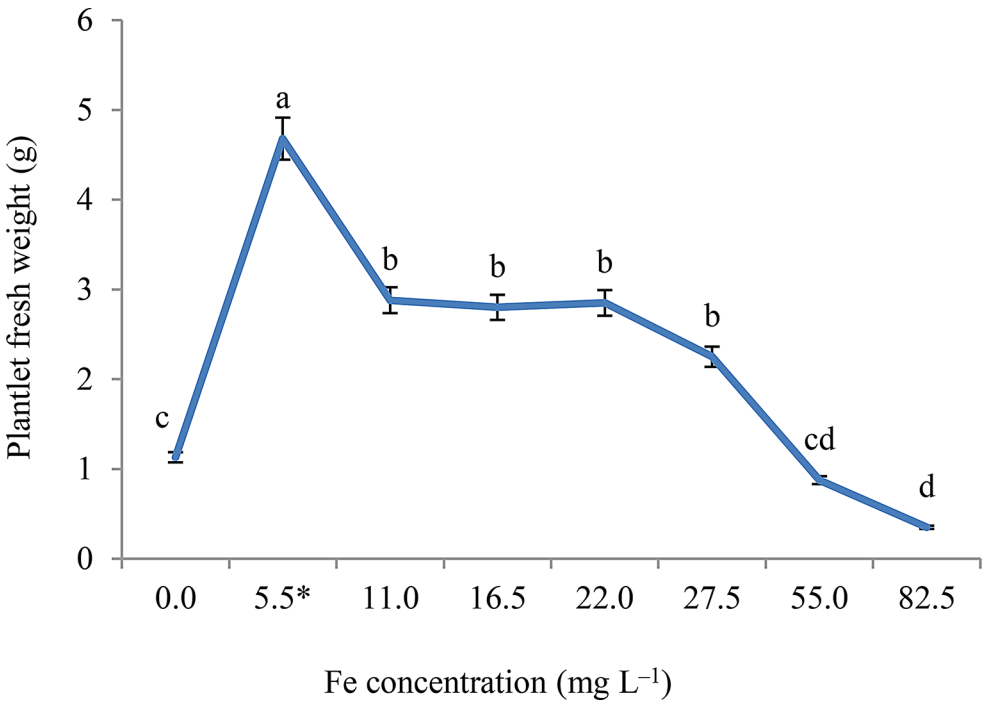

Fig. 2 Effect of different levels of iron (Fe) on fresh weight of "Williams" banana plantlets grown in in vitro. *= Concentration of Fe in standard Murashige and Skoog medium (MS). Values with the same letter (s) are not statistically different according to Duncan's multiple range tests

recorded with the highest Fe concentration (82.5 Fe). The concentration of 55.0 was as similar as the absence of Fe (0.0 mg L^{-1} Fe) for suppressing length of original shoot, plantlet fresh weight, plantlet dry weight and SPAD. It should be noted that shoots were blackened and the developed growth was ceased then the meristem cells died when Fe reached 55.0 mg L^{-1} (Fig. 5). Also, the highest Fe concentration (82.5 mg L^{-1}) led to growth arrest of plantlets hence, chlorophyll was not determined (Fig. 5).

3.2 Visual Symptoms of Fe Deficiency and Toxicity

The visual symptoms on banana plantlets were examined after two re-culture (about three months of the first re-culture). Nevertheless, plantlets of all Fe treatments gave different appearance of Fe deficiency and toxicity (Fig. 5).

Removing Fe (0.0 mg L^{-1} Fe treatment) from the MS medium caused Fe deficiency and the most common symptom is chlorosis of the entire lamina, then all plantlets turned

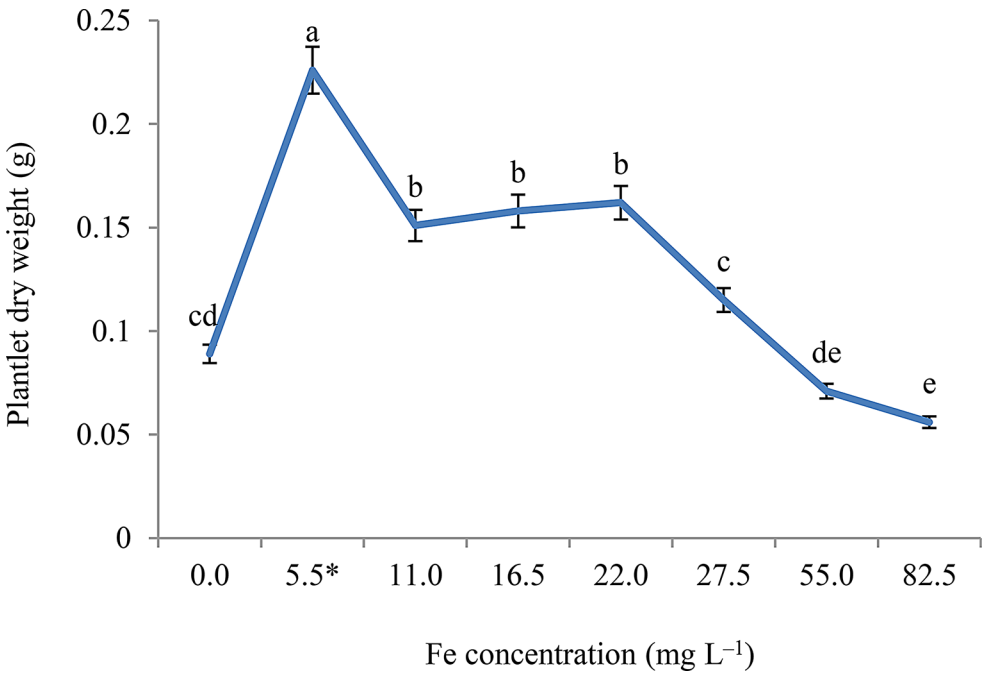

Fig. 3 Effect of different levels of iron (Fe) on dry weight of "Williams" banana plantlets grown in in vitro. *= Concentration of Fe in standard Murashige and Skoog medium (MS). Values with the same letter (s) are not statistically different according to Duncan's multiple range tests

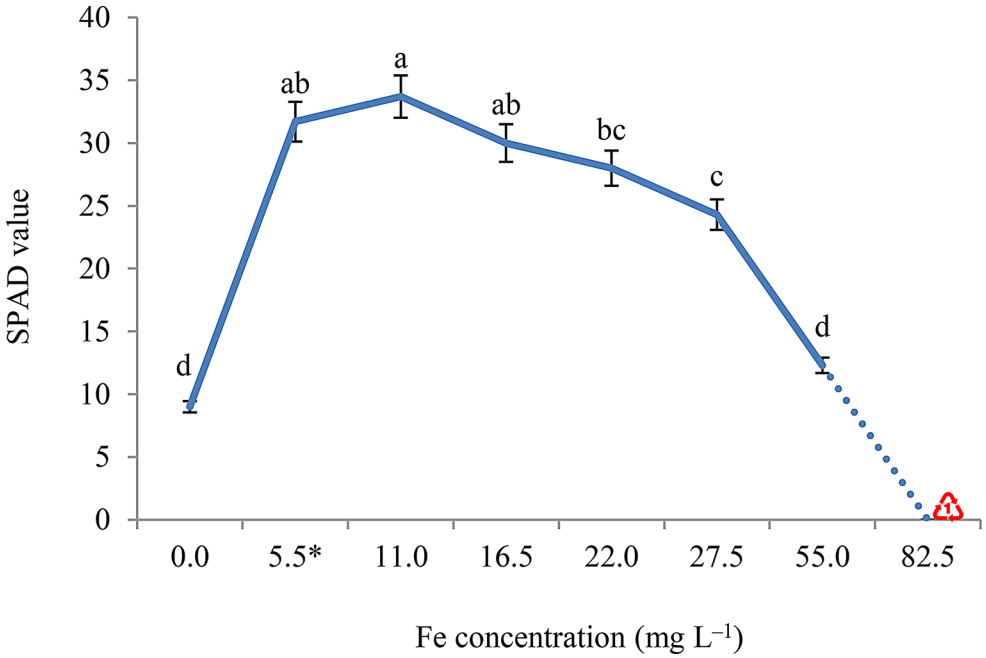

Fig. 4 Effect of different levels of iron (Fe) on relative chlorophyll content (SPAD) of "Williams" banana plantlets grown in in vitro. *= Concentration of Fe in standard Murashige and Skoog medium (MS). = Chlorophyll was impossible to be estimated, as plantlets grown in a medium containing 82.5 mg L^{-1} Fe occurred blackening and growth ceased. Values with the same letter (s) are not statistically different according to Duncan's multiple range tests

yellow or white and the medium became black in color (Fig. 6). On the contrary, more increase in Fe concentration in MS medium (82.5 mg L^{-1}) tended to blacken the shoots (Fig. 6). This means that Fe reach to toxicity level in plant tissue.

3.3 Macronutrient Content and Uptake

Results in (Table 2) showed that macronutrients (N, P and K) content and uptake in banana plantlet were influenced significantly by the Fe concentration in MS medium. Treatments of 11.0, 16.5 and 22.0 mg L^{-1} Fe gave the highest values of N and K (Table 2) content without noticeable differences among them. Removing Fe (0.0 mg L^{-1} Fe) or adding Fe at 5.5 or 27.5 mg L^{-1} (for N content), in addition to 55 or 85.5 mg L^{-1} (for K) gave similar values. There was non-significant variation among all Fe concentrations for P content, except application of Fe at 82.5 mg L^{-1}, which recorded the lowest value (Table 2).

Concerning the macronutrient uptake, the highest significant values of N and P uptake were obtained in the basic MS medium (5.50 mg L^{-1} Fe). In general, increasing Fe

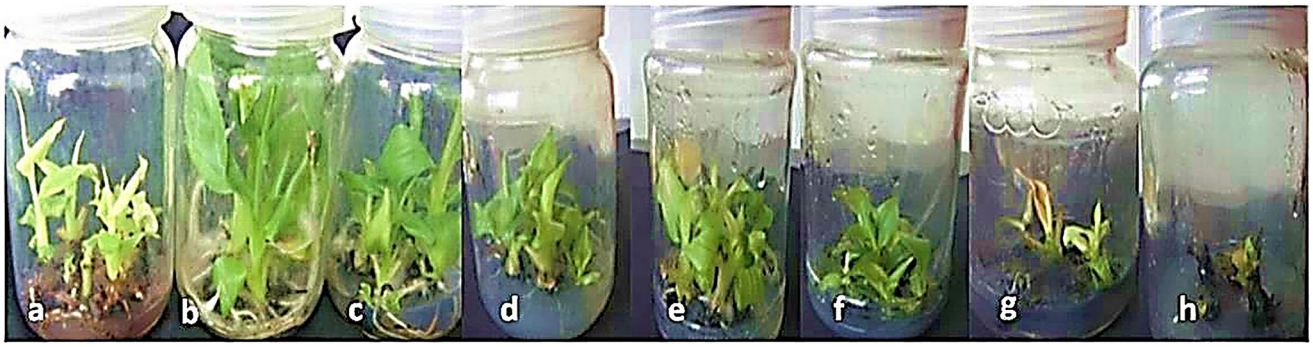

Fig. 5 Effect of different levels of iron (Fe) on "Williams" banana plantlets grown in vitro. (**a**, **b**, **c**, **d**, **e**, **f**, **g**, and **h**): Fe concentrations at 0.0, 5.5, 11.0, 16.5, 22.0, 27.5, 55.0 and 82.5 mg L^{-1}, respectively

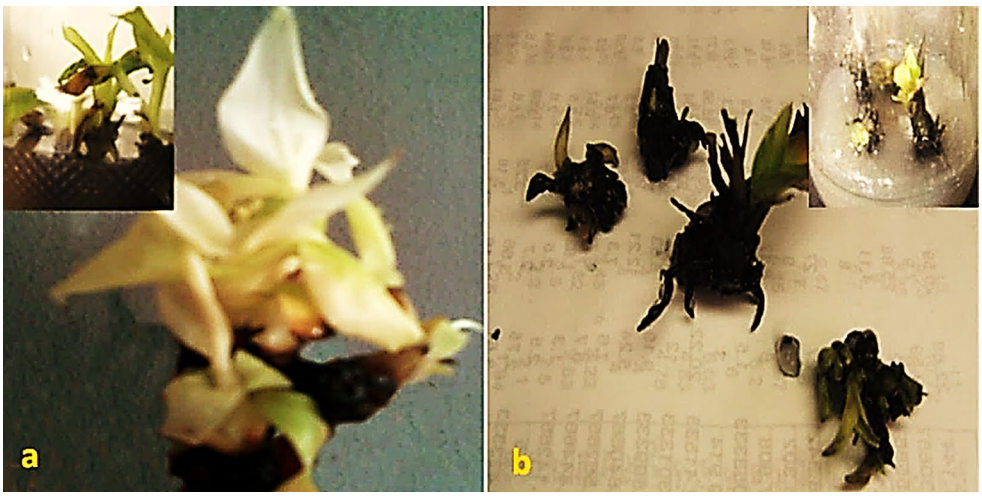

Fig. 6 Effect of iron (Fe) deficiency, 0.0 mg L^{-1} Fe (**a**) and toxicity, 85.5 mg L^{-1} Fe (**b**) on plantlets of "Williams" banana grown in vitro

Table 2 Effect of different levels of iron (Fe) on content and uptake of nitrogen (N), phosphorus (P) and potassium (K) in "Williams" banana plantlets grown in vitro

Fe (mg L^{-1})	Nutrient content (%)			Nutrient uptake (mg/plantlet)		
	N	P	K	N	P	K
0.0	2.77bc	0.449a	3.14b	2.47e	0.400 cd	2.79e
5.50 (MS)*	2.70c	0.400ab	3.86b	6.10a	0.904a	8.72c
11.0	2.99ab	0.347ab	6.05a	4.51c	0.524bc	9.14b
16.5	2.89a-c	0.431a	6.15a	4.57c	0.681b	9.72a
22.0	3.06a	0.339ab	5.89a	4.96b	0.549b	9.54ab
27.5	2.71c	0.314ab	4.06b	3.12d	0.361d	4.67d
55.0	2.20d	0.325ab	3.61b	1.56f	0.231de	2.56e
82.5	2.20d	0.292b	2.61b	1.23 g	0.164e	1.46f

*= Concentration of Fe in standard Murashige and Skoog medium (MS). Values in the same column followed by the same letter (s) are not statistically different according to Duncan's multiple range tests

concentration gradually in the medium decreased N and P uptake in the plantlets and the lowest significant value was obtained with 82.5 mg L^{-1} Fe treatment. On the other hand, removing Fe from the MS medium (0.0 mg L^{-1} Fe) reduced N and P uptake however it gave N values higher than the treatments of 55 and 82.5 mg L^{-1} Fe as well as P value higher than the treatment of 82.5 mg L^{-1} Fe. Banana plantlet grown in MS medium with 16.5 or 22.0 mg L^{-1} Fe contained the maximal values of K. While, increasing Fe concentration more than 22.0 mg L^{-1} reduced K uptake gradually and the least value was obtained by the highest Fe concentration (82.5 mg L^{-1}). Plantlets grown in Fe free medium had low K uptake resembles those of 55.0 mg L^{-1} Fe treatment.

Table 3 Effect of different levels of iron (Fe) on content and uptake of Fe, zinc (Zn) and manganese (Mn) in "Williams" banana plantlets grown in vitro

Fe (mg L^{-1})	Nutrient content (mg L^{-1})			Nutrient uptake (µg $plantlet^{-1}$)		
	Fe	Zn	Mn	Fe	Zn	Mn
0.0	50 g	117 a	133bc	4.45 h	10.4c	11.8d
5.50 (MS)*	320f	97 ab	157bc	72.3 g	21.9a	35.5bc
11.0	597e	83a-c	300ab	90.1f	12.5b	45.3ab
16.5	943d	80bc	367a	149.0e	12.6b	58.0a
22.0	1284c	53 cd	123c	208.0b	8.6d	20.0 cd
27.5	2116b	33d	97c	243.3a	3.8e	11.2d
55.0	2332b	37d	90c	165.6d	2.6f	6.4d
82.5	3034a	35d	63c	170.0c	2.0 g	3.5d

*= Concentration of Fe in standard Murashige and Skoog medium (MS). Values in the same column followed by the same letter (s) are not statistically different according to Duncan's multiple range tests

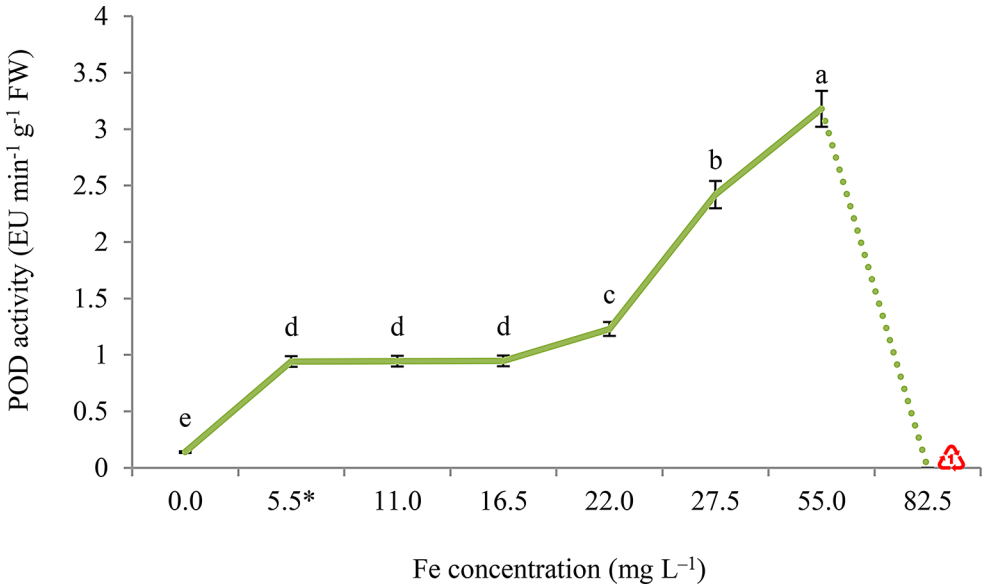

Fig. 7 Effect of different levels of iron (Fe) on peroxidase activity (POD) in the leaves extract of "Williams" banana plantlets grown in in vitro. *= Concentration of Fe in standard Murashige and Skoog medium (MS). = The activity of POD was impossible to be estimated, as plantlets grown in a medium containing 82.5 mg L^{-1} Fe occurred blackening and growth ceased. Values with the same letter (s) are not statistically different according to Duncan's multiple range tests

3.4 Micronutrient Content and Uptake

The content and uptake of all assessed micronutrients in banana plantlets significantly changed with different Fe concentrations in MS medium (Table 3). Fe content was increased gradually by the increase in Fe concentration in the media. Accordingly, the lowest value was obtained in plantlets grown in free Fe medium and the highest one was in plantlets grown in medium supplied with 82.5 mg L^{-1} Fe. The highest value of Zn was obtained by removing Fe from the medium. Zn content was gradually decreased by increasing Fe concentration up to 22.0 mg L^{-1} Fe. Thereafter, Zn content remained low but stable between 33 and 37 mg L^{-1} with treatments of 27.5, 55.0 and 82.5 mg L^{-1} Fe. With respect to Mn, MS medium received 16.5 or 11.0 mg L^{-1} Fe produced plantlets having the highest values of Mn content, surpassing Fe-free medium by 2.75 and 2.25 folds, respectively. The other treatments showed Mn values as similar as the treatment of 0.0 mg L^{-1} Fe.

As for micronutrients uptake, the treatment of 27.5 mg L^{-1} (for Fe uptake), 5.5 mg L^{-1} (for Zn uptake) and 16.5 or 11.0 mg L^{-1} (for Mn uptake) revealed the highest values (Table 3). Media with 0.0 mg L^{-1} Fe and 82.5 mg L^{-1} Fe showed the lowest values of Fe and Zn uptake in banana plantlets, respectively. Mn uptake looks like less affected by increasing the applied Fe concentrations above 16.5 mg L^{-1}, since the values obtained with 22.0, 27.5, 55.0 and 82.5 were significantly equal.

3.5 Activity and Isozyme Fingerprints for Peroxidase

Peroxidase activity as a metabolic indicator for Fe content was significantly affected by Fe treatments (Fig. 7). Removing Fe from medium gave the lowest value of POD activity. However, POD activity was increased gradually by the increase in Fe concentration in the media. The activity remained stable in plantlets grown in media with 5.5, 11.0 and 16.5 mg L^{-1} Fe. Thereafter, a sharp increase in activity

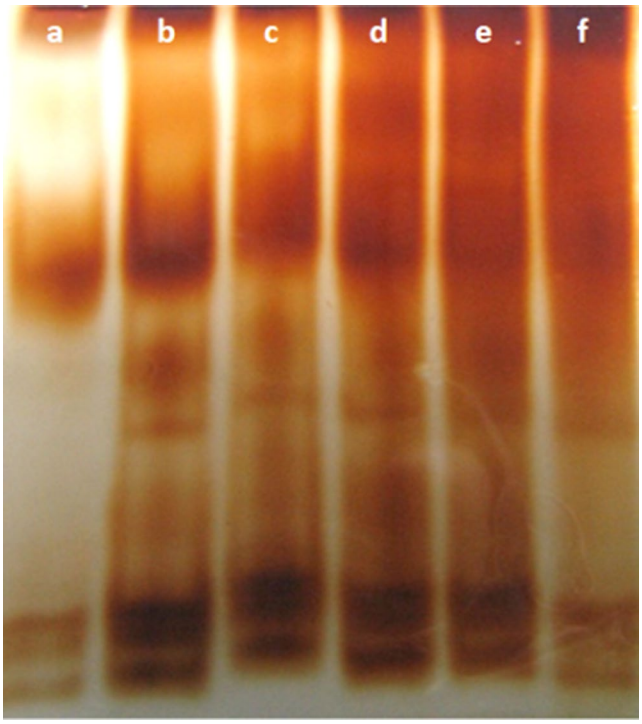

Fig. 8 Effect of different levels of iron (Fe) on electrophoretic patterns of peroxidase isozymes in the leaves extract of "Williams" banana plantlets grown in vitro. (**a**, **b**, **c**, **d**, **e** and **f**): Fe concentrations at 0.0, 5.5, 11.0, 22.0, 27.5 and 55.0 mg L^{-1}, respectively

of POD was observed in plantlets grown in medium with 22.0 mg L^{-1} Fe. This increase continued to reach the maximum value in plantlets grown in the medium supported with the highest Fe concentration (55 mg L^{-1} Fe). Whereas, plantlets grown in medium with 82.5 mg L^{-1} Fe were blackened and the growth was arrested, therefore it was impossible to estimate the POD activity.

The electrophoretic patterns and densitometry analysis of POD isozymes extracted from the leaves of "Williams" banana plantlets grown in in vitro under different levels of Fe in the media are illustrated in Fig. 8; Table 4. The obtained results revealed that there were six POD bands. However, these bands were not completely present in all Fe treatments. These six bands were detected in plantlets grown in media supported with 5.5, 11, 22, 27.5 and 55 mg L^{-1} Fe. Plantlets grown in medium free from Fe, gave only four bands where bands no.2 and 3 disappeared. The electrophoretic patterns of POD isozymes exhibited differences in density among different Fe treatments. The treatments of 5.50, 22.0, 27.5 and 55.0 mg L^{-1} Fe in most cases, showed high density of POD isozymes compared with 0.0 mg L^{-1} Fe.

4 Discussion

Certainly, the appropriate application of nutrients for crop plans is the major practice to avoid element deficiency and toxicity (Saudy 2015; Saudy et al. 2018; El-Metwally and Saudy 2021; Saudy and El-Metwally 2023). In this concern, micronutrients application had distinctive potentiality in keeping plant production, specifically in low fertile soils (Saudy et al. 2021; Shaaban et al. 2023). Fe contributes in physiological performance and mechanism of numerous processes in plants (Meharg 2012). Fe as a trace element had a pivotal role in the activity of plant growth processes such as respiration, synthesis of proteins, chlorophyll formation and replication of DNA (Takanori et al. 2018). For optimal growth, plants require Fe at concentration of 10^{-4}–10^{-9} M (Kim and Guerinot 2007). Since chloroplasts in majority of plant leaves comprise approximately 80% of Fe (Finazzi et al. 2015), Fe deficiency caused substantial reduction of chloroplast and chlorophyll concentration, with riven ultrastructure (Ding et al. 2016; Dey et al. 2021; Riaz and Guerinot 2021). Accordingly, banana plantlets grown in medium free of Fe showed weak growth expressed in low shoot length and plantlet weights. In this respect, Cinelli et al. (2003) carried out an in vitro culture experiment on quince and pear and found that fresh and dry weights were reduced under Fe limiting conditions. Moreover chlorophyll concentration was declined by the reduction of Fe supply into the culture medium compared to control medium. Electron transfer involved in photosynthesis process is influenced by Fe, thus deficiency of Fe can hinder water photolysis step, reducing the photosynthetic rate (Singh et al. 2005). On the other hand, over-uptake of Fe can cause toxicity,

Table 4 Effect of different levels of iron (Fe) on densitometry of peroxidase isozymes in the leaves extract of "Williams" banana plantlets grown in vitro

Band number	Relative mobility	Fe concentration (mg L^{-1}) in the media					
		0.0	5.50 (MS)*	11.0	22.0	27.5	55.0
1	0.363	+	++	++	+++	+++	+++
2	0.495	×	++	++	+++	+++	+++
3	0.615	×	+	+	+	+	+
4	0.730	+	++	++	++	++	+
5	0.883	++	+++	+++	+++	+++	++
6	0.952	++	+++	+++	+++	+++	++

*= Concentration of Fe in standard Murashige and Skoog medium (MS). ×= Absent, + = Low density, ++= Moderate density, +++ = high density

damaging cell membrane and plant growth, eventually ultimately impairing plant health (Kumar et al. 2010). Herein, in MS media having high Fe concentrations, specifically at 55.0 and 82.5 mg L^{-1}, substantial reductions in growth with absence of chlorophyll were noticed. In this concern, the green biomass of plantlet turned into black in medium having 55.0 mg L^{-1} Fe, while 100% degradation in chlorophyll was observed in medium having 82.5 mg L^{-1} Fe. Toxicity of Fe could cause infirm cell growth while reducing nutrients uptake, chlorophyll content and activity of photosynthesis process (de Oliveira Jucoski et al. 2013; Peña-Olmos et al. 2014). Due to severe Fe toxicity, necrosis of leaf was recorded and plant died (Zahra et al. 2021b). Also, yellowing and chlorosis symptoms were observed owing to Fe toxicity (Meharg 2012). Physiologically, Fe toxicity stimulated the oxidative stress, damaging the plasma membrane due to over generation of reactive oxygen species (ROS). Proteins, lipids, and DNA are dramatically damaged by ROS causing cell death (Onyango et al. 2018). The drastic levels of Fe^{2+} ions increased the generation of ROS, which are overmuch toxic, inducing the damage of DNA and carbohydrate, while increasing lipid peroxidation and protein oxidation, hence cell death (Boruah and Bharali 2015). Furthermore, the toxicity of Fe resulted in increasing malondialdehyde (MDA), the main responsible of lipid peroxidation (Sinha and Saxena 2006).

As for the content of nutrients, current work has shown that inappropriate Fe concentration, especially a concentration higher than normal, creates confusion in the balance of nutrients. Excess Fe can also influence the uptake of essential nutrients, such as zinc and phosphorus, by interfering with the transporters that facilitate their uptake leading to nutritional imbalance, and reduced plant health (Meharg 2012). Fe toxicity had the potential to detrimentally impact the uptake and balance of other vital nutrients in plants (Kim et al. 2019; Dey et al. 2020). As well, it has been reported that excess Fe can alter the genes expression involved in Fe uptake, transport and homeostasis (Thomine and Vert 2013).

The current work also showed that banana plantlets suffered from oxidative stress owing to the toxicity of excess Fe concentration or Fe deficiency. However, plants can relatively adjust the metabolism of ROS that associated with oxidative stress via stimulating the activity of antioxidant system (El-Bially et al. 2022; Makhlouf et al. 2022; Ramadan et al. 2024b; Elshiekh et al. 2025). Herein, POD as an efficient scavenger foe ROS was more active under high Fe supply that caused toxicity symptoms. In flowering Chinese cabbage, the stress induced by Fe deficiency occurred disturbance in the metabolic system of ROS, causing a distinctive increase in POD activity and accumulation of MDA (Wang et al. 2022).

The symptom 'chlorosis' is appearing under deficit Fe conditions because of inhibiting the biosynthesis of chlorophyll and development of chloroplast (Selby-Pham et al. 2017; Ma et al. 2019). Elevating Fe concentration declined photosynthetic pigments, while increased the antioxidant enzyme activity (Delias et al. 2022). Fe plays a crucial role as cofactor for peroxidase enzyme, which participates in physio-biochemical events, involving respiration, photosynthesis, synthesis and repairing of nucleic acid, nutrient balance, and maintenance the integrity of proteins of plants (Mahender et al. 2019; Li et al. 2021). Also, in stressed conditions, phenolic compounds increased (Ali et al. 2024b; Emam et al. 2024; El-Ziat et al. 2024; Helal et al. 2024). This could interpret why the color of medium changed to be black. Due to the release of phenolic by the plantlets, the medium became black in color (Chen et al. 2024).

As shown in electrophoretic pattern, it seems that the differences in staining intensity were in general agreed with differences found in the activity of POD. Al-Shabi, (2002) indicated that POD isozymes was more efficient to study the genetic diversity for sensitive and tolerant sorghum cultivars for Fe deficiency and salt stress. Fe deficiency appears to influence various peroxidase isozymes to different degrees and leads to secondary oxidative stress, as evidenced by increased H_2O_2 levels (Ranieri et al. 2001). Salama et al. (2009) stated that there were alternations in POD isozyme profile under Fe stress.

5 Conclusions

In the present work, it could be summarized that there are a positive relationship between iron concentration in Murashige and Skoog medium and peroxidase activity or peroxidase isozymes. The molecular changes expressed in peroxidase activity and isozymes could be helpful in the diagnosis iron deficiency or toxicity impact on banana plantlets grown in vitro. however, for practical application at the production level to outstand the gain fertilization program for bananas in arid regions, further studies should be adopted and data collected from farms and linked to the results obtained in the current study.

Author Contributions Noha Mansour and Hani Saber Saudy: designed the experiments; Noha Mansour, Ibrahim. Shawky, Ahmed EL-Gazzar and Hani Saber Saudy: performed the experiments; Noha Mansour and Hani Saber Saudy: analyzed the data and wrote the manuscript. Ibrahim. Shawky and Ahmed EL-Gazzar: supervised and finalized the manuscript. All authors agree with the manuscript contents and with its submission.

Funding Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB).
Self-funded research.

Data Availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval The authors declare that all experiments were carried out per all ethical standards and regulations. Humans or animals were not used in any of the investigations.

Conflict of interest The authors declare no conflict of interest.

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