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Piper nigrum: Micropropagation, Antioxidative enzyme activities, and Chromatographic Fingerprint Analysis for Quality Control

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Abstract A reliable in vitro regeneration system for the economical and medicinally important *Piper nigrum* L. has been established. Callus and shoot regeneration was encouraged from leaf portions on Murashige and Skoog (MS) medium augmented with varied concentrations of plant growth regulators. A higher callus production (90 %) was observed in explants incubated on MS medium incorporated with 1.0 mgL^{-1} 6-benzyladenine (BA) along with 0.5 mgL^{-1} gibberellic acid after 4 weeks of culture. Moreover, a callogenic response of 85 % was also recorded for 1.0 mgL^{-1} BA in combination with 0.25 mgL^{-1} α-naphthalene acetic acid (NAA) and 0.25 mgL^{-1} 2,4-dichlorophenoxyacetic acid or 0.5 mgL^{-1} indole butyric acid (IBA) along with 0.25 mgL^{-1} NAA and indole acetic acid. Subsequent sub-culturing of callus after 4 weeks of culture onto MS medium supplemented with 1.5 mgL^{-1} thiodiazoran or 1.5 mgL^{-1} IBA induced 100 % shoot response. Rooted plantlets were achieved on medium containing varied concentrations of auxins. The antioxidative enzyme activities [superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX)] revealed that significantly higher SOD was observed in regenerated plantlets than in other tissues. However, POD, CAT, and APX were higher in callus than in other tissues. A high-performance liquid chromatography (HPLC) fingerprint analysis protocol was established for quality control in different in vitro-regenerated tissues of *P. nigrum* L. During analysis, most of the common peaks represent the active principle "piperine." The chemical contents, especially piperine, showed variation from callus culture to whole plantlet regeneration. Based on the deviation in chromatographic peaks, the in vitro-regenerated plantlets exhibit a nearly similar piperine profile to acclimated plantlets. The in vitro regeneration system and HPLC fingerprint analysis established here brought a novel approach to the quality control of in vitro plantlets, producing metabolites of interest with substantial applications for the conservation of germplasm.

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Abbreviations

TDZ	Thiodiazoran
BA	6-Benzyladenine
GA_3	Gibberellic acid
NAA	α-Naphthalene acetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
IBA	Indole butyric acid
IAA	Indole acetic acid
MS0	MS medium without plant growth regulators
PGRs	Plant growth regulators
SOD	Superoxide dismutase
POD	Peroxidase
CAT	Catalase
APX	Ascorbate peroxidase
WHO	World Health Organization
RSD	Relative standard deviation

Introduction

Piper nigrum L. (black pepper), famous as the king of spices, is an active member of the family "Piperaceae" [1], is native to India, and is mostly cultivated in tropical and subtropical regions [2]. *P. nigrum* is extensively used in food cooking and perfumery. The quality of peppercorn can be judged from its pungency contributed by the active component piperine [3–5]. *P. nigrum* is not restricted to human dietaries, but can be used for multiple purposes including medicinal applications and chemical defenses [6]. Two important compounds of *P. nigrum*, viz., β-caryophyllene and nerolidol, have anesthetic activity [7], flavoring, and is presently under investigation as skin enhancer for the transportation of therapeutic drugs through transdermal activity [8].

Conventionally, *P. nigrum* is propagated through cuttings rather than seeds due to its heterozygous nature. In the last few years, a substantial degree of decline has been noticed in production through the attack of several pathogens like mycoplasma, bacteria, virus, fungi, and pests [1, 2]. To eliminate and address such problems, biotechnological methods like plant tissue culture meet the increasing demand for production. The exploitation of in vitro culture techniques has offered the opportunity to regenerate plants from somatic tissues bypassing the sexual barrier [9]. These cells regenerate and may result in genetically stable and useful genotypes. In the cited literatures, little evidence on *P. nigrum* regeneration exists from leaf tissues. However, multiple regeneration systems from nodal and shoot tip explants have been documented [3, 4, 10, 11].

During plant organogenesis, sometimes, an unrestrained production of reactive oxygen species (ROS) can directly damage cells or through the production of toxic metabolites [6, 12]. To combat such oxidative stress, plants produce specific enzymes during different differentiation phases [13]. These enzymes form a complex antioxidant protection system such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) which scavenge both toxic free radicals and their associated non-radical oxygen species [9]. But the role of antioxidative enzymes during the organogenesis of

P. nigrum is little known. However, Abbasi et al. [9] reported the clear relationship of antioxidative enzyme production during organogenesis in *B. rapa*. Vijayakumar et al. [14] have documented the effect of supplementation of peppercorn on tissue lipid peroxidation and enzymatic and non-enzymatic antioxidants in high-fat diet-fed rats and concluded that this spice can reduce high-fat diet-induced oxidative stress. *P. nigrum* also protect the intestine against induced oxidative stress; inhibit lipid peroxidation and the quenching of superoxides and hydroxyl radicals by piperine; minimize induced lung carcinogenesis; and inhibit human lipoxygenase [14–18].

Micropropagation-based quality control in medicinal species performs an encouraging role to guarantee the protection and efficiency of crude drugs before final pharmaceutical product formation [19]. The World Health Organization (WHO) provides broad guidelines starting from precise authentication to the post-harvest processing of materials (World Health Organization 2003). Fingerprint analysis for quality control was announced and acknowledged by the WHO as an approach for the investigation of plant-based product and to standardize traditional knowledge of medicines. In vitro cultures of valuable medicinal species offer dependable quality control and existence without any environmental changes. But the commercialization of active compounds needs applicable techniques for awareness and to enhance productivity. Quality control for in vitro-regenerated plantlets mostly emphasizes on the morphological observation and the extraction of mother compounds, whereas fingerprint analysis provides basic categorization of a complex system of plants with a calculable degree of consistency. High-performance liquid chromatography (HPLC) is a reliable technique for active compound determination and for fingerprint analysis of medicinal plant species [19]. In the literature cited and to the best of our knowledge, no HPLC-based chromatographic fingerprinting of *P. nigrum* L. has been reported. Therefore, the overall objectives of the present experimental work is to establish a reliable system for the in vitro regeneration of *P. nigrum* L. and to develop chromatographic fingerprints for the quality control of micropropagated tissues as well as the determination of the most bioactive component, piperine, in propagated plants.

Materials and Methods

Plant Materials

Seeds were provided by Assist. Prof. Bin Guo, Key Laboratory of Resource Biology and Biotechnology in Western China (Northwest University), Ministry of Education; School of Life Science, Northwest University, Xi'an 710069, China. Seeds were surface-decontaminated according to the modified method of Abbasi et al. [9]. Seeds were first exposed to 75 % ethanol for 2 min followed by dipping in 2 % mercuric chloride for 30 s. The seeds were then thoroughly washed three times with double autoclaved water. The seeds' viability was checked upon dipping in autoclaved water. The seeds floating on the water's surface were discarded. The viable seeds were then divided into two groups: one exposed to concentrated sulfuric acid while the latter was exposed to gibberellic acid (GA_3) solution. Seeds from both groups were incubated in Murashige and Skoog (MS) medium without plant growth regulators (PGRs) or supplemented with 0.5 mgL^{-1} of GA_3. For best germination, the seeds were kept at 32 °C in an incubator for 15–37 days.

In Vitro Cultures

Suitable leaf explants were selected from germinated seedlings of *P. nigrum*. These leaf explants were surface-decontaminated using the method of Abbasi et al. [20]. Uncut leaves were exposed to 0.05 % (*w*/*v*) mercuric chloride ($HgCl_2$) for ~2 min and 70 % (*v*/*v*) ethanol solution for 60 s. These sterilized leaves were then thoroughly washed with double autoclave distilled water. For whole regeneration, MS [21] medium was used containing 30 g/L sucrose (Merck) and 8 g/L agar (Sigma). Before agar addition and pH adjustment (~5.8), different cytokinins, auxins, and thiodiazoran (TDZ) were supplemented to the medium. Different media were autoclaved (121 °C) for 20 min and then maintained under in vitro growth conditions at ~25±3 °C with the required photoperiod. Reasonable leaf explant pieces (~3–4 mm^2) were cut for callus induction and shoot organogenesis. For different parameters on callus induction and organogenesis, the MS-basal medium was augmented with 6-benzyladenine (BA) alone (0.5–2.0 mgL^{-1}) or either BA in combination with gibberellic acid (GA_3) or in combination with 2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthalene acetic acid (NAA), indole butyric acid (IBA) alone or in combination with NAA and indole acetic acid (IAA) or GA_3 with or without BA and various concentrations of TDZ (0.5, 1.0–4.0 mgL^{-1}). MS medium without PGRs (MS0) was used as a control for whole regeneration. Five or six explants were incubated in 100-mL Erlenmeyer flask containing ~25 to ~35 mL of the medium. Data regarding callus induction (in percent) were recorded after ~4 weeks of explant incubation. A reasonable amount of friable callus was shifted to the MS medium for shoot organogenesis with similar compositions of PGRs. Data on percent shoot response was documented after 4 weeks of callus cultures under similar growth conditions. In vitro shoots with suitable length were carried to the next medium for rooting containing different concentrations (0.5, 1.0, 1.5, and 2.0 mgL^{-1}) of IBA alone or IBA with different concentrations of NAA (0.25, 0.5, 1.0, and 1.5 mgL^{-1}) and IAA, BA, with NAA and 2,4-D after 6 weeks; rooted plantlets were carefully removed from the medium, washed several times with sterile distilled water, and transferred to a soil mixture for further growth under controlled conditions of greenhouse. Callus was collected after 4 weeks; in vitro shoots and plantlets were collected after 30 days of culture. Each plant material was dried in an oven at 50 °C before HPLC analysis. However, for antioxidant enzyme activities, fresh tissues were used.

Antioxidant Enzyme Activities

Fresh tissues of *P. nigrum* L. were used for antioxidative enzyme activities. Tissue extracts were prepared using the method of Meratan et al. [22], with slight modifications. Before the activities, ice-cold 0.5 M Tris–HCl and buffer (pH 6.8) were used for homogenate extraction of callus, in vitro shoots, and regenerated plantlets. Subsequently, the resulted extracts were centrifuged for 20 min at 10,000 rpm at 4 °C to retain enzyme viability. For accurate results, the collected supernatant after centrifugation was preserved in a freezer before enzyme activities. Agilent spectrophotometer (USA) was used for the absorption of different extracts. For the determination of SOD, a well-established method of Giannopolitis and Ries [23] was used. For CAT and POD assays, the methods of Arrigoni et al. [24] and Abeles and Biles [25] were followed. APX was determined according to the method of Miyake et al. [26].

"Piperine" Extraction and HPLC Analysis

The extraction of "piperine" from different tissues of *P. nigrum* L. was determined according to the method of Liu et al. [19]. In vitro-regenerated tissues were oven-

dried and then powdered for fingerprint analysis and piperine quantification. Of the powered materials, 140 mg was extracted with 25 mL methanol in a 50-mL volumetric flask. The plant materials were then slightly heated in a water bath for 15 min. But the results were not satisfactory. Then, the extractions for each plant tissues were made through a Soxhlet system for 48 h. The extracts were then filtered through 45-μm membrane before HPLC analysis. The HPLC system (Shimadzu Lc8A, Japan) was set with a binary pump, a variable wavelength (λ) detector, solvent vacuum degasser, and an autosampler with a 10-μL injection loop. The column was C18 (ODS) with a 25×4.6-mm, 5-μm particle size. For buffer preparation, accurately 1.36 g of potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in HPLC grade water (900 mL). The pH of the buffer was adjusted to 2.8 with the addition of dilute phosphoric acid (H_3PO_4) and the volume made up to 1 L with HPLC grade water. The final solution was filtered through a 45-μm membrane filter and then the mobile phase prepared (A75/B25) as (A) methanol (75) and (B) buffer (25). Before usage, the solution was degassed. The injection volume was 10 μL and the effluent was monitored at 254 nm by a UV detector with a flow rate of 1.5 mL/min. Piperine standard (Sigma) was prepared by taking 25 mg of piperine into a 50-mL flask, shaking well, and the final volume made with methanol. Accurately 10 μL piperine standard was injected into the HPLC. Chromatogram and the retention time for piperine were recorded. A similar amount of the standard was again injected for 5 min to find the relative standard deviation (RSD) of the area. Similarly, 10-μL samples were injected and compared with the retention time of the piperine standard; the percentage of piperine in samples was determined according to the formula

$$\%\ \text{Piperine(w/w)} = [\text{AS}_\text{M}/\text{AS}_\text{T} \times \text{wt of } \text{S}_\text{T}/50 \times 50/\text{wt of } \text{S}_\text{M} \times \text{purity of } \text{S}_\text{T}/100 \times 100]$$

where A is the area of the standard and sample, S_M represents the sample, and S_T represents the standard.

Statistical Analysis

All experiments were designed in complete randomized block and each treatment consisted of nine replicates in Erlenmeyer flasks. For accurate statistical analysis, each experiment was repeated twice. All the data were analyzed using ANOVA, and Duncan's multiple range test was used for comparisons among means. However, for antioxidative enzyme activities and for chromatographic fingerprinting, data were collected from triplicates and represented as the mean±SD.

Results and Discussion

In Vitro Regeneration of *P. nigrum*

During regeneration, endogenous contamination caused severe setback to the in vitro establishment of *P. nigrum* [3, 4]. For the decontamination of in vitro cultures, we used the valuable protocol of Abbasi et al. [1, 20] and Ahmad et al. [2, 27]. The application of these protocols significantly abridged the contamination up to ~80 %. Many protocols for leaf explant sterilization showed sensitivity to various reagents [28–31]. However, we did not observe any inhibitory effect of the decontamination protocol on regeneration.

All PGRs used influenced callus induction followed by shoot organogenesis and, finally, rooting (Fig. 1). Callogenesis is believed to be feasible product of indirect organogenesis for active compound accumulation in many medicinal plant species [1, 12, 13]. The effects of various PGRs such as BA with 2,4-D (0.25, 0.5, 1.0, and 1.5 mgL^{-1}) and NAA or various concentrations of BA alone or 1.0 mgL^{-1} constant BA in combination with GA_3 or GA_3 alone, IBA alone or either in combination with IAA (0.25, 0.5, 1.0, and 1.5 mgL^{-1}) or NAA, and different concentrations of TDZ on indirect organogenesis were evaluated (Fig. 2). The leaf explants of *P. nigrum* in the present study responded to all PGRs applied (Fig. 2). The best callus induction was recorded on MS medium supplemented with 1.0 mgl^{-1} BA (90 %) in combination with 0.5 mgl^{-1} GA_3 (Fig. 2). A similar effect of PGRs was recently reported by Abbasi et al. [1] in *Silybum marianum* regeneration. Furthermore, 85 % callus response was documented for either BA in combination with NAA and 2,4-D (0.25 mgL^{-1}) or 0.5 mgL^{-1} IBA with 0.25 mgL^{-1} IAA and NAA, respectively. The current data are an agreement with the observations of Abbasi et al. [9] that BA in combination with NAA produced maximum callus in *B. rapa*. A lower amount of callus response was observed for various combinations of IBA and GA_3. Callus induction recorded for BA at different concentrations was significantly higher than most of the PGRs used, and no callus was observed on MS0 medium. A similar observation—that BA alone or along with GA_3 promotes callus production in *P. nigrum*—was previously reported by Ahmad et al. [2]. Callus response >70 % was recorded for 3.0 and 4.0 mgL^{-1} TDZ, but a lower concentration of TDZ induced a lower amount of callus (Fig. 2). The combination of cytokinins and auxins influences callus to maximum levels, but lower than cytokinins alone. Bhat et al. [3] documented that leaf explants from *P. nigrum* showed no clear response to a similar composition of PGRs; however, other species of *Piper* showed a similar response. Data reported by Philip et al. [4] regarding the regeneration of *P. nigrum* from the shoot tip are in agreement with the current data. Madhusudhanan and Rahiman [32] mentioned the effect of activated charcoal that influences callus production, but is unable to regenerate plantlets from leaf explants.

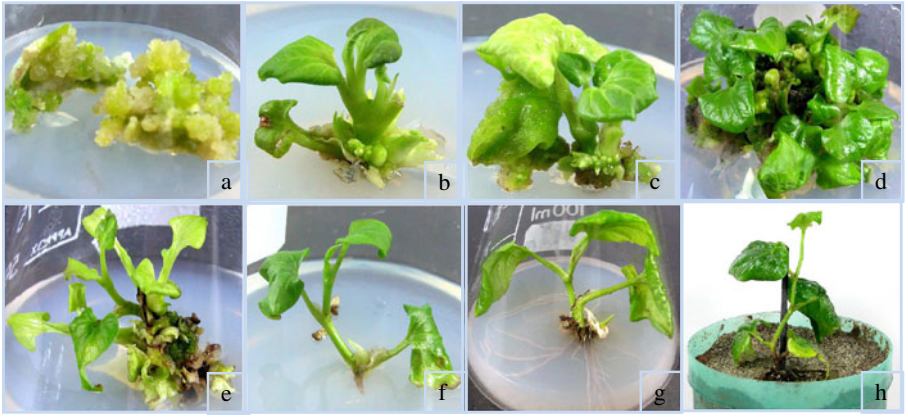

Fig. 1 In vitro regeneration of black pepper (*P. nigrum*) from leaf explants. **a** Callus induction on a medium containing 1.0 mgL^{-1} BA in combination with 0.5 mgL^{-1} GA_3. **b**, **c** Shoot regeneration from callus subculture. **d** Shoot proliferation on shoot organogenesis medium. **e** Shoot elongation. **f** Root initiation. **g** Root formation and plantlet maturation. **h** Acclimatization of in vitro-regenerated plantlets

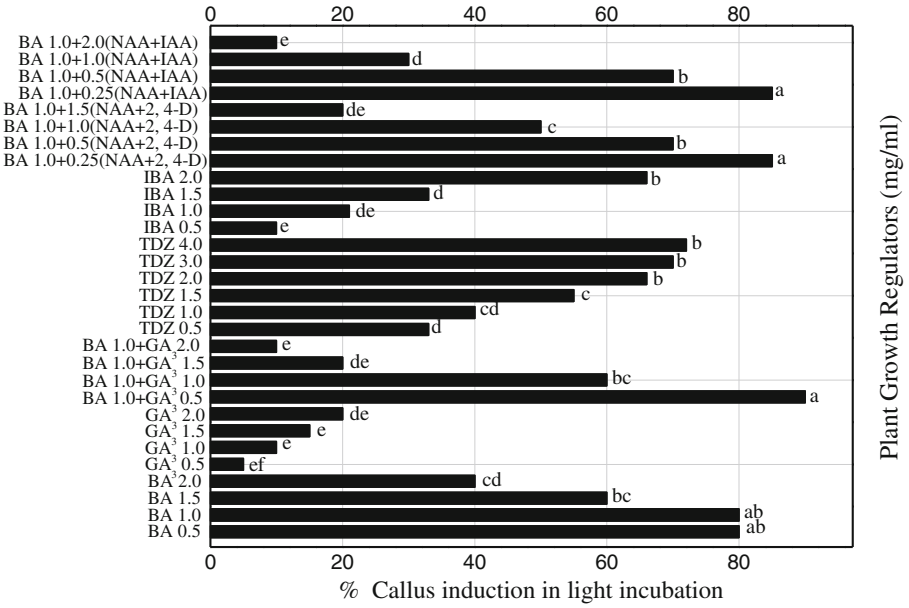

Fig. 2 Effects of various concentrations of BA with 2,4-D (0.25, 0.5, 1.0, and 1.5 mgL^{-1}) and NAA or various concentrations of BA alone or 1.0 mgL^{-1} constant BA in combination with GA_3 or GA_3 alone, IBA alone in different concentrations or either in combination with IAA (0.25, 0.5, 1.0, and 1.5 mgL^{-1}) and NAA, and different concentrations of TDZ on percent callus induction in *P. nigrum* from leaf explants. Data were collected after 4 weeks of culture. Values are the means of three replicates. *Columns with common letters* are not significantly different at $P<0.05$.

Mature callus was shifted onto shoot organogenesis medium, and the data were documented after 30 days of sub-culture. The best percent shooting response (100 %) was recorded for 1.5 mgL^{-1} TDZ after 4 weeks of culture (Fig. 3). However, a lower concentration of TDZ (0.5 mgL^{-1}) influences 80 % shooting from leaf explants. As compared to TDZ alone, 1.5 mgL^{-1} IBA also promoted 100 % shooting response, where a higher concentration of IBA induced 85 % shoot response, which is greater than 0.5 and 1.0 mgL^{-1} IBA concentrations. In the current research work, all the BA concentrations (0.5, 1.0, 1.5, and 2.0 mgL^{-1}) used were effective in percent shoot induction; however, the BA-containing medium, when supplemented with 2,4-D and NAA, inhibits percent shooting significantly (Fig. 3). Similarly, Ahmad et al. [2] also found that BA was effective in shoot organogenesis in *P. nigrum*. Similar results were also reported by Ahmad et al. [27], where BA alone can significantly influence percent shooting response in *Stevia rebaudiana*. The current data are in agreement with the observation of Abbasi et al. [9, 20] in various medicinal plants species. Similar percent shooting was also reported in *Cardiospermum halicacabum* L., *Swerta chirata*, and *Ruta gravoelens* L. on BA- and TDZ-induced medium [33–35]. In the present investigation, the synergistic combination of IBA, NAA, and IAA significantly reduced percent shooting response in *P. nigrum*. Furthermore, various concentrations of GA_3 alone were also less effective in percent shoot induction.

The elongated shoots collected from shoot organogenesis were transferred to the rooting medium. Optimum percent rooting (88 %) was obtained for 1.5 mgL^{-1} of IBA; however, 85 % rooting was also recorded when the MS medium was incorporated with 0.5 mgL^{-1} IBA in combination with IAA and NAA (0.25 mgL^{-1}) after 30 days of culture (Fig. 4). Moreover, >80 % rooting was recorded for 2.0 mgL^{-1} IBA alone or IBA in various combinations with NAA and

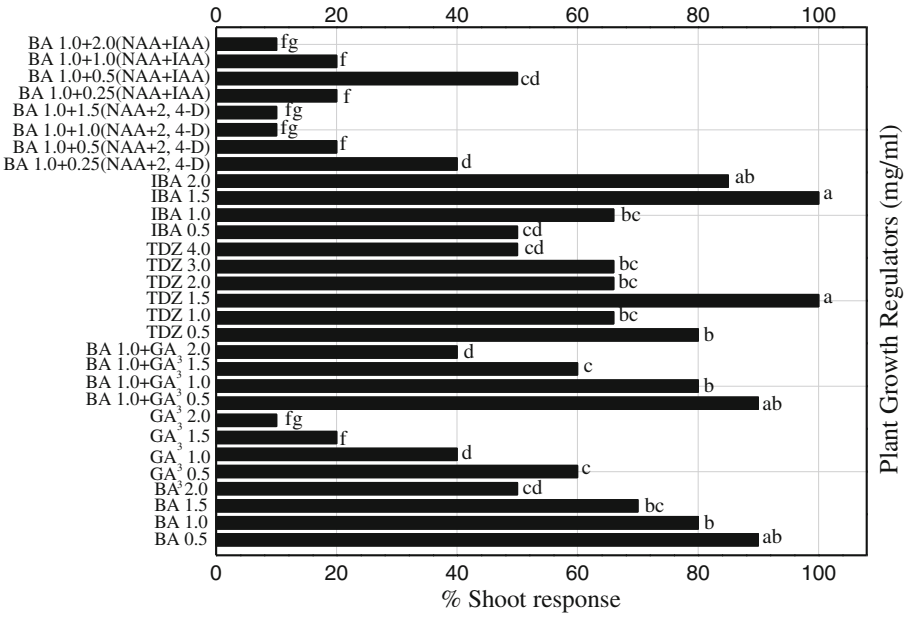

Fig. 3 Effects of various concentrations of BA with 2,4-D (0.25, 0.5, 1.0, and 1.5 mgL^{-1}) and NAA or various concentrations of BA alone or 1.0 mgL^{-1} constant BA in combination with GA_3 or GA_3 alone, IBA alone in different concentrations or either in combination with IAA (0.25, 0.5, 1.0, and 1.5 mgL^{-1}) and NAA, and different concentrations of TDZ on percent shoot induction in *P. nigrum* from leaf explants. Data were collected after 4 weeks of culture. Values are the means of three replicates. *Columns with common letters* are not significantly different at $P<0.05$.

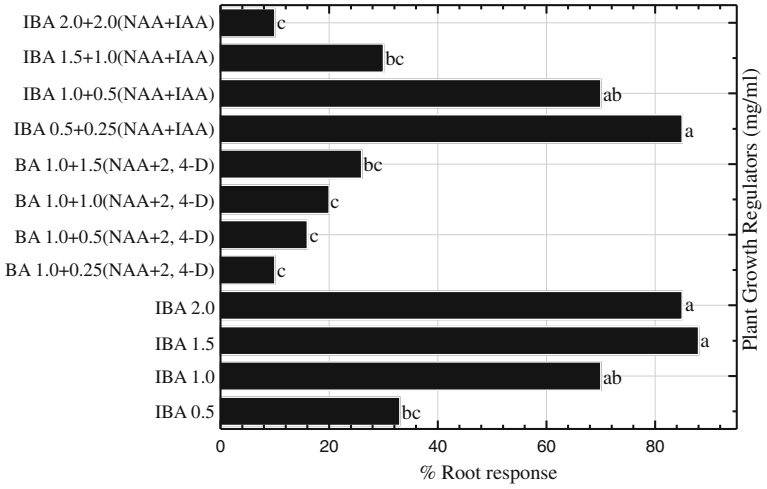

Fig. 4 Effects of different concentrations (0.5, 1.0, 1.5, and 2.0 mgL^{-1}) of IBA alone or IBA with different concentrations of NAA (0.25, 0.5, 1.0, and 1.5 mgL^{-1}) and IAA, BA with NAA and 2,4-D (0.25, 0.5, 1.0, and 1.5 mgL^{-1}) on percent rooting. Data were collected after 6 weeks of culture. Values are the means of three replicates. *Columns with common letters* are not significantly different at $P<0.05$.

IAA. Contrarily, Philip et al. [4] obtained rooted plantlet through shoot tip cultures of *P. nigrum* on 1 mgL^{-1} NAA. In another study on *Piper* spp., rooting was achieved by the addition of IAA [36]. In our experiments, we obtained healthy rooting on the different PGRs applied (Fig. 1g). *P. nigrum* is acclimatization-friendly, therefore can easily fit in both pot and field soil. Rooted plantlets were successfully transferred to pots for further growth which were maintained in controlled conditions (Fig. 1h).

Antioxidative Enzyme Activities

During in vitro conditions, different tissues of *P. nigrum* produced varied antioxidative enzymes to protect themselves from biotic and abiotic stresses, especially from oxidative stress. However, these enzymes are not specific to one type of tissue, but were produced in different quantities during organogenesis [9, 27]. In the present investigation, the activities of CAT, POD, and APX were found higher in callus than sprouted shoot and plantlets in *P. nigrum* (Table 1). The activity of SOD was higher in regenerated plantlets than callus and regenerated shoot. A similar observation in which SOD was higher in regenerated plantlets than in other tissues was also reported by Abbasi et al. [9] in *B. rapa*. An increase in CAT and POD production and a decrease in SOD production as observed during shoot organogenesis were also reported in *Prunus*, *Solanum*, and *Gladiolus* [37–39]. But here, we observed inverse activities: a decrease in CAT and POD and an increase in SOD during shoot organogenesis. Meratan et al. [22] reported that calli secreted CAT during callus differentiation, while SOD increases during the organogenesis of *Acanthophyllum sordidum*. SODs and CAT have a pivotal role in the cellular regulation of hydrogen peroxide concentration. SODs function in the removal of the superoxide radical, simultaneously producing H_2O_2 as a reaction product [40]. POD and CAT were high in the callus of *P. nigrum*, while SOD was higher in regenerated plantlets than in callus. APX was higher in callus, but as the callus enters into the organogenesis phase, the activity becomes lower. However, when the regenerated shoot starts rooting, the activity again increases. In the current experiment, it was clearly observed that in vitro cultures of *P. nigrum* are able to produce secondary metabolites in quantities more than that of the original parts of the plant. Moreover, when callus cultures enter into shoot and root organogenesis, variable quantities of metabolites were produced containing phenolic and flavonoid compounds. These compounds formed a complex network of antioxidants which control ROS, which is a strong oxidizer that causes the damage of major biological molecules: proteins, lipids, and nucleic acids. The production of SOD, POD, CAT, and APX fluctuates during the different growth phases and is highly dependent upon explant types. The fluctuation in these antioxidant enzyme activities

Table 1 Activities of SOD, POD, CAT, APX (in units per milligram protein) in callus, regenerated shoots, and regenerated plantlets of *P. nigrum* L.

Plant tissues	SOD	POD	CAT	APX
Callus	0.716±0.02b	0.046±0.001a	0.039±0.001a	0.072±0.002a
Regenerated shoots	0.776±0.03b	0.026±0.001ab	0.037±0.001a	0.027±0.001b
Regenerated plantlets	1.476±0.05a	0.008±0.002c	0.012±0.001b	0.032±0.001b

Values are the means of three replicates. Means with the same letter within each column are not significantly different at $P<0.05$

SOD superoxide dismutase, *POD* peroxidase, *CAT* catalase, *APX* ascorbate peroxidase

in the regenerated tissues of *P. nigrum* may be due to changes in the oxidative gene-encoding activities. Such studies will elucidate the relationship between antioxidant enzyme activities and in vitro cultures of valuable species such as *P. nigrum*.

Chromatographic Fingerprint of Tissue Culture Plants

After successful in vitro regeneration, callus, in vitro shoots, plantlets, and acclimated *Piper* were collected at various growth stages for chromatographic fingerprint analysis. To standardize the fingerprints of tissue cultured materials through HPLC, similar peaks present in all samples were denoted as "common peaks" for piperine production in *P. nigrum* L. The chromatographic data along with the piperine standard represent valuable information for the assessment and identification of *P. nigrum* L. The fingerprint chromatogram of all in vitro and in vivo *Piper* samples, and the authentication of HPLC fingerprinting was completely based on the relative peak area and retention time. For fingerprint development in *P. nigrum*, the active principle "piperine" was selected as the reference compound. The resulting "common peaks," relative peak areas, and relative retention times were achieved in comparison with the reference substance. The RDS must be in the range of 1 %. If the RSD of the common peaks in *Piper* samples is <1 % (Table 2), these peaks can be considered as similar compounds. In the present research work, the RSD of the relative retention times is <1.0 % for five common peaks, which indicates the presence of the same compound in different samples. Furthermore, if the RSD of the relative peak area of common peaks is higher than 10 %, we recommend that the compound is different from the standard one. Bioactive "piperine" in different samples of *P. nigrum* showed ascending variation from callus culture to plantlet development. Especially acclimated plantlets after in vitro regeneration produced nearly similar contents of piperine as compared to in vitro plantlets. These findings can support the use of chromatographic fingerprint analysis in the quality control of tissue cultured *P. nigrum* and can also be used for other valuable plant species.

Quality Evaluation of Tissue Cultured Plants

HPLC is an ideal technique for the qualitative and quantitative assessment of bioactive compounds in medicinal plants. For the quality control of *P. nigrum*, validated HPLC methods for piperine estimation are summarized with the required parameters, including injection volume, flow rate of the mobile phase, the column used, detector, and the mobile phase. Presently, the chromatogram clearly revealed that the piperine contents increased from callus culture to plant development, which started from >0.8 % in callus to >0.18 % in plantlets and finally reaching 0.42 % in acclimated plantlets, with good correspondence to each other, as shown in Table 2. The results based on dry weight (DW) showed that callus

Table 2 Relative retention time, relative standard deviation (RSD) and piperine content in callus, in vitro shoots, in vitro plantlets and acclimated plantlets in *P. nigrum* L

Piper tissues	Relative retention time	RSD (%)	Piperine contents ($mg g^{-1}$ DW)
Callus	9.1	0.0852	0.852
In vitro shoot (4 weeks)	8.7	0.1082	1.08
In vitro plantlets (4 weeks)	9.4	0.1644	1.64
Acclimated plants	7.6	0.4163	4.16

Values presented in the table are the means of three replicates

produced 0.852 mgg^{-1} DW piperine, in vitro shoot produced 1.08 mgg^{-1} DW, in vitro plantlets produced 1.64 mgg^{-1} DW, while a higher amount of piperine (4.16 mgg^{-1} DW) was detected in acclimated plantlets (Table 2). Piperine estimation in different regenerated tissues showed that the organogenesis phase like shoots and roots accumulate more contents than the callus culture. These displayed results provide enough information for the quality control and chromatographic fingerprinting of piperine in different tissues and also the correct time of collection. Whole tissues of *P. nigrum* have been harvested for therapeutically active piperine and for other pharmaceutical preparations. In conclusion, a reliable in vitro regeneration system along with HPLC fingerprint analysis for quality control established here is suitable for germplasm conservation and uniform production of this medicinally important *P. nigrum* L.

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