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Original Article

Effect of Plant Growth Regulators and Light on Callus Induction, Antioxidant Enzyme Response and *Total Phenol* in *Nigella arvensis* L.

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ABSTRACT

The tissue culture of *Nigella arvensis* L. was carried out on an MS medium supplemented with various concentrations of auxins and cytokinins. Media fortified with different concentrations (0.0, 0.5, 1.0 and 1.5 mg/L) of 2, 4-D, NAA or IBA in combination with kinetin or BAP (1.5 mg/L and 2 mg/L) were used to induce and grow callus in present and absent of light. The experimental design was performed as a randomized block factorial design with three replications. Following callus culture, antioxidant enzymes activity and total phenolic contents were evaluated. The maximum callus induction (80.9%) was obtained with 1.0 mg/L 2, 4-D and 1.5 mg/L kinetin in the presence of light. Plant growth regulators combination and concentration, as well as photoperiod, affected the activity of antioxidant enzymes. The highest activities of catalase and peroxidase and total phenolic contents were measured in media with BAP combinations. On the contrary, the activity of superoxide dismutase was at the least in all BAP combinations. Overall, light intensified the activities of enzymes, but it had a negative effect on total phenolic contents.

INTRODUCTION

Traditionally, medicinal herbs have been used to treat and prevent human diseases [1]. The interest in medicinal plants and their secondary metabolites is owing to their high content of phenols, alkaloids and terpenoids. These phytochemicals have been used as functional food and drug products [2].

The *Nigella* genus has a long history of domestication, they wildly grow in Asia Europe and North Africa [3,4]. This genus is popularly known as black seed or black cumin and belongs to the *Ranunculaceae* family and includes species such as *N. arvensis*, *N. sativa* and *N. oxypetala* Boiss. [5]. The seeds of the plant are used as ingredients in several foods such as bread, pickle and cookies. In addition, these plants have been utilized as medicinal plants to treat different ailments. The extracts of black seeds have s carminative, antispasmodic, anti-inflammatory, antidiabetic, antibacterial, and antitumor properties [6-9].

Phytochemical investigations on the genus *N. arvensis* led to the isolation of several active compounds such as different classes of flavonoids, terpenoids, proteins, alkaloids and triterpene glycosides [10]. The medicinal value of the plants depends on bioactive metabolites constituents that lead to certain physiological functions in the human body. Phenolic components are one of the most

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important antioxidants among plant bio-products [11-12].

The development of plant tissue cultures to produce secondary metabolites has been considered in recent decades. Plant cell cultures offer promising potential alternative sources to produce pharmaceutical agents of industrial importance. This platform is a powerful production system for plant secondary metabolites grad less of restrictions caused by geographical and seasonal variation as well as the constraints of access to rare or endangered species. Among diverse plant cell culture approaches, callus culture is the appropriate method f to produce various bioactive compounds and healthy plant material for propagation [13-14].

Plant growth regulators (PGRs) as one of the most influential components of culture media control cell growth, development and morphogenesis and metabolite formation [15]. Furthermore, light is an important environmental factor affecting cell growth, differentiation the formation of plant products including both primary and secondary metabolites [16]. Reactive oxygen species (ROS) are important signaling compounds of major processes in plants involved in the development and producing metabolites. Furthermore, these radicals can start chain reactions such as lipid peroxidation, oxidizing DNA, and proteins, leading to cellular damage [17]. Plants have defense systems to scavenge cellular ROS by non-enzymatic and enzymatic systems [18]. In this research, the effect of different supplementations of growth regulators such as Naphthaleneacetic acid (NAA) and 6-Benzylaminopurine (BAP) on the callus induction of *N. arvensis*, antioxidant enzyme activity (catalase, peroxidase and superoxide dismutase) and total phenol content in the present and absent of light was studied.

MATERIAL AND METHODS

Seeds of *N. arvensis* were purchased from Pakanbazr Isfahan (Iran). The seeds were surface sterilized using sodium hypochlorite and cultured in a *half*-time Murashige and Skoog *(*MS) medium [19]. Fresh young leaves from three weeks old plants were used for callus induction. The explants of leaves were cultured on a solidified MS medium enriched with auxin PGRs (2, 4-Dichlorophenoxyacetic acid, Naphthaleneacetic acid and Indole-3-butyric acid) and cytokinin PGRs (Kinetin and 6-Benzylaminopurine).

The experimental design was performed as a randomized block factorial design (6 × 6 × 2) which the first factor (a) was a different combination of auxin and cytokinin (2,4-D × KN, 2,4-D × BAP, NAA × KN, NAA × BAP, IBA × KN, IBA × BAP). Another factor (b) was the different concentrations of hormones in which 2,4-D, NAA and BAP were used in three concentrations of 0.5, 1.0 and 1.5 mg/L while KN and BAP were applied in the concentrations of 1.5 and 2.0 mg/L. The third factor (c) was two different photoperiod conditions: darkness and photoperiod lighting (16 hours of light and 8 hours of darkness). Three replications were considered for this experiment and each experimental unit consisted of three explants per tissue culture container. The explants were grown at 25 ± 1 mg/L, and the resulting callus tissues were sub-cultured every three weeks under the same media and growth conditions. Duncan's multiple range test was used to test differences among the means. The percentage of callus induction, catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) activity and the phenolic content were measured in grown calli.

The fresh callus samples were homogenized in pre-chilled mortar and pestle in extraction phosphate buffer [20]. A crude extract was prepared in an extraction buffer containing 1% polyvinyl pyrrolidone in 100 mM sodium phosphate buffer (0.1 mol/L, pH 6.8). The homogenate was centrifuged at 12000 *g* for 20 minutes at 4 °C, and the supernatants were used for the following enzymatic activity assay. The protein concentration of various extracts and solutions was quantified according to the method of Bradford [20]. This crude extract was used to measure the activity of enzymes.

Catalase (CAT) activity was assayed by monitoring the decomposition of H_20_2 by measuring the decrease in absorbance at 240 nm of a reaction mixture described by Golamnezhad et al [21]. In brief, the assay mixture contained 2.6 mL of sodium phosphate buffer (pH 6.8), 3% H2O2, and 0.1 ml of the enzyme extract. Changes in absorbance were read at 240 nm. Enzyme activity in terms of µmoles hydrogen peroxide consumed per min per mg of protein was calculated using the molar extinction

coefficient of 43.6 M–1cm–1 for hydrogen peroxide.

The peroxidase activity was quantified based on the change in absorbance at 500 nm due to oxidation of phenylenediamine (PPD, benzene-1,4-diamine) in the presence of hydrogen peroxide [22]. Reaction mixture in total volume (3.0 ml) consisted of 10 mM H_2O_2 in 50 mM phosphate buffer (pH 6.8), 0.1 ml of PPD (100mM) and 0.1 ml of the enzyme extract. The POD activity was estimated by the increase in the absorbance of the product at 500 nm for one minute at room temperature.

Total superoxide dismutase activity was determined by measuring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT)**.** The assay mixture contained 1.9 ml sodium phosphate buffer, 0.1 ml EDTA (1 mM), 0.25 ml riboflavin (2mM), 0.25 ml L-methionine (13mM) and 0.25 ml NBT (75mM). Then, 0.1 ml of the enzyme extract was added to the reaction mixture in glass test tubes and illuminated by 3 sets of 10-W fluorescent tubes for 20 minutes. The absorbance was read at 560 nm in the spectrophotometer against the blank [23]. The percentage of O_2 scavenging activity was calculated as follows:

O_2 scavenging activity % = [($OD_{Control}$–OD_{Sample})/ $OD_{Control}$]*100%

One unit of enzyme activity was defined as the quantity of enzyme causing 50% inhibition of the NBT photochemical reduction.

Methanol 80% was used to extract the phenolic components. Total phenolic contents were determined using the Folin–Ciocalteu reagent assay. Additionally, 1 ml of the extract was diluted with 4 ml of distilled water and 0.5 ml of the Folin–Ciocalteu reagent was added. After 5 minutes 1 ml of a 5% Na_2CO_3 solution was added and mixed. The mixture was kept in the dark at room temperature for 60 min [24-25]. Gallic acid was used to generate a standard curve. The absorbance of each callus extract and Gallic acid was monitored at 760 nm.

Data were analyzed using the general linear model (GLM) procedure in the SAS software version 9.4 (SAS Institute, Cary, NC, USA). Duncan's test at the $P \leq 0.05$ significance level was used to compare the means.

RESULTS

After 10 to 20 days of culture, callus formation of leaf explants was observed. Analysis of variance indicated that the main effects of PGRs, concentration, light and their interactions were significant ($P<0.01$) for the callus induction under *in vitro* conditions (Table 1).

In general, 2,4-D at high concentrations (1.0 and 1.5 mg/L), along with KN and BAP under both dark and light conditions, had the greatest effect on the induction of callus formation. However, the comparison of the means using Duncan's test indicated that the highest percentage of callus formation was obtained in the use of 1.0 mg/L of 2,4-D and 1.5 mg/L of KN under photoperiod conditions (M = 80.9, SD = 3.14). There was no significant difference with some hormonal treatments, especially those containing 1.0 or 1.5 mg/L 2,4-D (Table 2). Moreover, the lowest rate of callus induction (M = 32.6, SD = 8.68) was in a culture medium containing 2 mg/L KN and 1.5 mg/L IBA in the dark ($P<0.05$), which was not significantly different from some treatments containing various concentrations of NAA (Table 2).

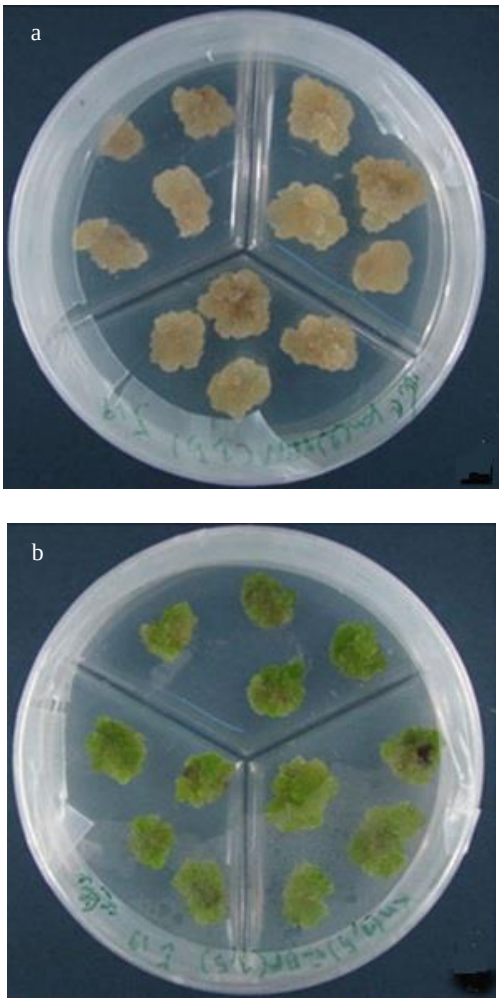

Fig. 1 The nature of *N. arvensis* L. callus grown in different conditions of light. a: darkness, b: photoperiod regime (16 h light and 8 h dark).

The appearance of calluses was different in the exposure to light and darkness. The results demonstrated that the callus remained creamish yellow or white under dark conditions (Fig. 1-A) while in the photoperiodic regime the color of calluses became pigmented and turned green or whitish green (Fig. 1-B).

The activity of CAT, SOD and POD enzymes was assayed using the spectrophotometric method. Analysis of variance indicated that the main effects of PGRs, concentration, light and their interactions were significant (*P<0.01*) for the antioxidant enzymes activity and total phenolic contents in the calluses grown in a culture medium, except light for CAT enzyme activity (Table 1). Duncan's multiple range test indicated that mean of CAT enzyme activity for the BAP×NAA treatment (M = 4.3171, SD = 2.880) mean was significantly different from that of other PGRs combinations (Table 3) except BAP×IBA (M = 4.0671, SD = 1.946). Similarly, the highest POD activity of the calluses grown in the culture medium was observed in the BAP×NAA treatment (M = 42.782, SD = 15.005) which was significantly different from that of other hormonal treatments (Table 3). The lowest activity of the POD hormone, which was significantly different from that of other hormonal treatments, was detected in KN×2,4-D (M = 23.315, SD = 11.251). Contrary to CAT and POD, the lowest mean values of SOD activity were observed in the treatments containing BAP hormone combination (M = 4.964, SD = 1.992), which showed a significant difference with other treatments consisting of the KN hormone in their culture medium (M = 7.465, SD = 2.985). Most activities measured for SOD were in KN×2,4-D (M = 7.950, SD = 2.447) with a significant difference from those of other hormonal treatments (Table 3). In the present study, the results demonstrated that the highest mean of phenol content (M = 170.086, SD = 54.635) was observed in a medium containing a combination of BAP-IBA, which was significantly different from that of other hormonal treatments (Table 3). The Mean comparison of data also showed that the lowest values of phenol content were obtained in treatments containing KN hormone (Table 3).

There was no significant difference in the activity of CAT under contrasting photoperiodic regimes. On contrary, light exposure led to a decrease in total phenolic contents, so that the total phenolic content measured in calli under dark conditions (M= 103.694, SD = 50.058) was significantly higher than that under light-dark photoperiod conditions (M= 99.740, SD = 49.159). However, in all of the above cases, different concentrations of the PGRs, as well as the type of PGRs, caused different reactions to the light treatments (Fig. 2). Figure 2 presents a detailed distribution of enzyme activity and total phenolic contents in different conditions, including the type and concentration of PGRs as well as the presence or absence of light.

Exposure to light had a different effect on hormonal activity and phenol content of calli (Table 4). The mean comparison applying the Duncan's test (*P<0.05*) revealed that the amount of POD (M = 37.717, SD = 18.283) and SOD (M = 6.616, SD = 3.456) activity under light-dark photoperiod conditions was significantly different from that under dark conditions (Table 4).

Table 1 Analysis of variance for percentage of callus induction, activity of antioxidant enzymes and total phenol content of *N. arvensis* L. callus in different PGRs.

Source of variation	DF.	F Value callus induction	CAT	POD	SOD	Total phenolic contents
PGRs	5	37.14 **	26.27 **	189.50 **	1976.32 **	2139.97 **
Concentration	5	21.70 **	25.62 **	95.90 **	235.73 **	5687.66 **
Light	1	16.08 **	0.35 ns	961.68 **	33.41 **	2142.16 **
PGRs ×Con	25	11.12 **	18.98 **	55.27 **	186.14 **	4387.10 **
PGRs ×Light	5	6.03 **	16.09 **	180.06 **	187.14 **	3588.97 **
Con × Light	5	4.50 **	45.60 **	9.07 *	125.12 **	2966.07 **
PGRs × Con × Light	25	5.70 **	19.79 **	74.70 **	104.00 **	2728.97 **
Error	142	-	-	-	-	-
Total	215	-	-	-	-	-
Coeff. Var	-	10.0	24.3	9.3	3.2	4.9

*: Significant at 5% probability level, **: Significant at 1% probability level, ns: Non-significant

Table 2 Effect of growth regulators and light conditions on percentage of callus induction *N. arvensis* L.

		Light / KN		Dark / KN		Light / BAP		Dark / BAP	
		1.5 (mg/L)	2.0 (mg/L)	1.5 (mg/L)	2 (mg/L)	1.5 (mg/L)	2.0 (mg/L)	1.5(mg/L)	2(mg/L)
2,4-D	0.5 (mg/L)	68.8 ± 5.40 b-j	61.9 ± 1.87 g-n	63.8 ± 6.23 e-m	44.7 ± 7.60 t-x	66.6 ± 6.07 c-k	55.7 ± 5.63 m-s	75 ± 8.10 a-c	59.5 ± 3.77 j-p
	1.0 (mg/L)	80.9 ± 3.14 a	70.6 ± 4.69 b-h	76.2 ± 4.31 ab	70.8 ± 5.15 b-g	63.3 ± 2.36 e-m	74.4 ± 5.12 a-d	75.2 ± 14.46 a-c	60.3 ± 3.99 j-o
	1.5 (mg/L)	66.4 ± 3.29 c-k	69 ± 7.44 b-j	52.9 ± 6.21 n-t	73.6 ± 3.70 a-d	71.4 ± 5.69 a-g	77.1 ± 4.72 a-b	56.1 ± 5.17 l-s	70 ± 5.53 b-h
IBA	0.5 (mg/L)	49.2 ± 7.86 q-v	52.3 ± 3.97 o-u	58.8 ± 6.32 k-p	61.2±5.41 h-o	57.1 ± 4.72 l-r	58.7 ± 7.92 k-q	49 ± 3.35 r-v	62.3 ± 5.53 f-n
	1.0 (mg/L)	71.4 ± 3.50 a-g	74.6 ± 2.49 a-d	66 ± 7.57 c-i	65.3±5.17 e-l	63.4 ± 5.45 e-m	72.3 ± 4.69 a-e	61.1 ± 2.98 h-o	63.9 ± 5.97 e-m
	1.5 (mg/L)	47.6 ± 3.37 s-x	57.1 ± 7.28 k-s	32.6 ± 8.68 y	22.8±9.50 z	52.3 ± 7.37 o-u	56.1 ± 3.64 l-s	53. 3± 6.76 o-t	57.3 ± 3.83 k-r
NAA	0.5 (mg/L)	60.7 ± 6.46 i-o	59 ± 4.46 k-p	47.8 ± 6.60 r-w	61.2 ± 4.62 h-o	52.8 ± 7.09 n-t	41. 9 ± 5.26 v-y	55.5 ± 5.63 m-s	42.8 ± 2.21 u-x
	1.0 (mg/L)	65.8 ± 9.43 c-k	61.9 ± 5.37 g-n	52.9 ± 5.57 n-t	41.2 ± 8.41 v-y	55.5 ± 7.47 m-s	50.0 ± 6.46 p-v	57.1 ± 5.93 k-s	38.5 ± 2.93 w-y
	1.5 (mg/L)	47.6 ± 9.12 s-x	61.1 ± 5.73 h-o	38.2 ± 2.82 xy	72.1 ± 0.82 a-e	52.3 ± 8.85 o-u	50.7 ± 7.12 p-v	69.9 ± 3.12 b-h	71.8 ± 2.17 a-f

Each data represents the mean of three replicates ± standard deviation of the representative traits (SD).

*: Values followed by different letters are significantly different according to a Duncan's test at $P< 0.05$

Table 3 Mean of antioxidant enzymes activity and total phenol content of *N. arvensis* L. callus grown in media with different plant growth factors

Hormone	Mean of enzyme activity or phenol content			
	CAT	POD	SOD	Phenol content
	U/mg Protein	U/mg Protein	U/mg Protein	Mg/g FW
Kn × 2,4-D	3.2627 ± 3.754 b	23.3152 ± 11.251 f	7.95011 ± 2.447 a	66.815 ± 20.605 e
Kn × IBA	1.283 ± 0.2832 c	32.3039± 20.925 c	7.75931 ± 3.516 b	75.926 ± 15.117 d
Kn × NAA	3.4528 ± 1.026 b	30.3100 ± 20.236 d	6.68636 ± 2.895 c	74.696 ± 19.803 d
BAP × 2,4-D	2.5443 ± 1.247 c	26.6378 ± 11.802 e	4.71042 ± 2.621 e	107.272 ± 45.910 c
BAP × IBA	4.0671 ± 1.946 a	33.8819 ± 8.797 b	5.59791 ± 1.532 d	170.086 ± 54.635 a
BAP × NAA	4.3171 ± 2.880 a	42.7818 ± 15.005 a	4.58236 ± 1.640 f	115.509 ± 34.786 b

Each data represents the mean of three replicates ± standard deviation of the representative traits (SD).
*: Values followed by different letters are significantly different according to a Duncan's test at $P < 0.05$

Table 4 Mean of antioxidant enzymes activity and total phenol content of *N. arvensis* L. callus grown in different light conditions

Photoperiod	Mean of activity of content			
	CAT	POD	SOD	Phenol content
	U/mg Protein	U/mg Protein	U/mg Protein	Mg/g FW
Light	3.3616 ± 2.121 a	37.7166 ± 18.283 a	6.61597 ± 3.456 a	99.7403 ± 49.159 b
Dark	3.4281 ± 2.509 a	25.3603 ± 11.300 b	5.81285 ± 2.012 b	103.6943 ± 50.058 a

Each data represents the mean of three replicates ± standard deviation of the representative traits (SD).
*: Values followed by different letters are significantly different according to a Duncan's test at $P< 0.05$

DISCUSSION

Modern plant biotechnological approaches such as plant cells and tissue cultures are significant alternative sources to the whole plant to produce high-value bioactive compounds. Successful production of high yields of secondary compounds in plant cell cultures has been reported frequently [26-28]. In this research, multiple combinations of cytokinin and auxin were used for callus induction. In addition, the effect of light exposure on the formation of callus was investigated. The amount of callus induction was associated with the growth regulator combination and the photoperiodic condition. Callus induction is achieved by the application and modulation of growth regulators either by stimulating endogenous hormones or by applying exogenous growth regulators to the nutrient medium [29]. The rate of callus induction can vary depending on the type and concentration of exogenous growth regulators [30,31]. A combination of 1.0 mg/L 2,4-D and 1.5 mg/L KN was reported as most suitable for callus induction from leave explants of *N. sativa* (100% callus induction) in the light conditions of 16 h photoperiod [32]. In another study, the best callus induction (82.6%) media for *N. sativa* was determined in the presence of 1.0 mg/L NAA and 1.0 mg/L KN [33]. In *Aconitum heterophyllum, another member of the Ranunculaceae* family the maximum callus induction was reported to be 70% in a medium supplemented by 0.6 mg/L NAA and 1.0 mg/L BAP [34]. The amount of callus induction in the light was higher than in the darkness. In *Gossypium hirsutum* L., the rate and speed of callus formation were remarkably higher in light-treated explants than in dark-treated ones [35]. The best callogenic response in *Silybum marianum* L. was reported in the combination of NAA and BAP in photoperiod [36]. However, there are some conflicting reports about the effect of light on callus growth. In *Cnidium officinal*, the callus grown under dark condition exhibited maximum biomass [37]. This impact also has been observed in *Ajuga bracteosa* [38] and *Taraxacum officinale* [39].

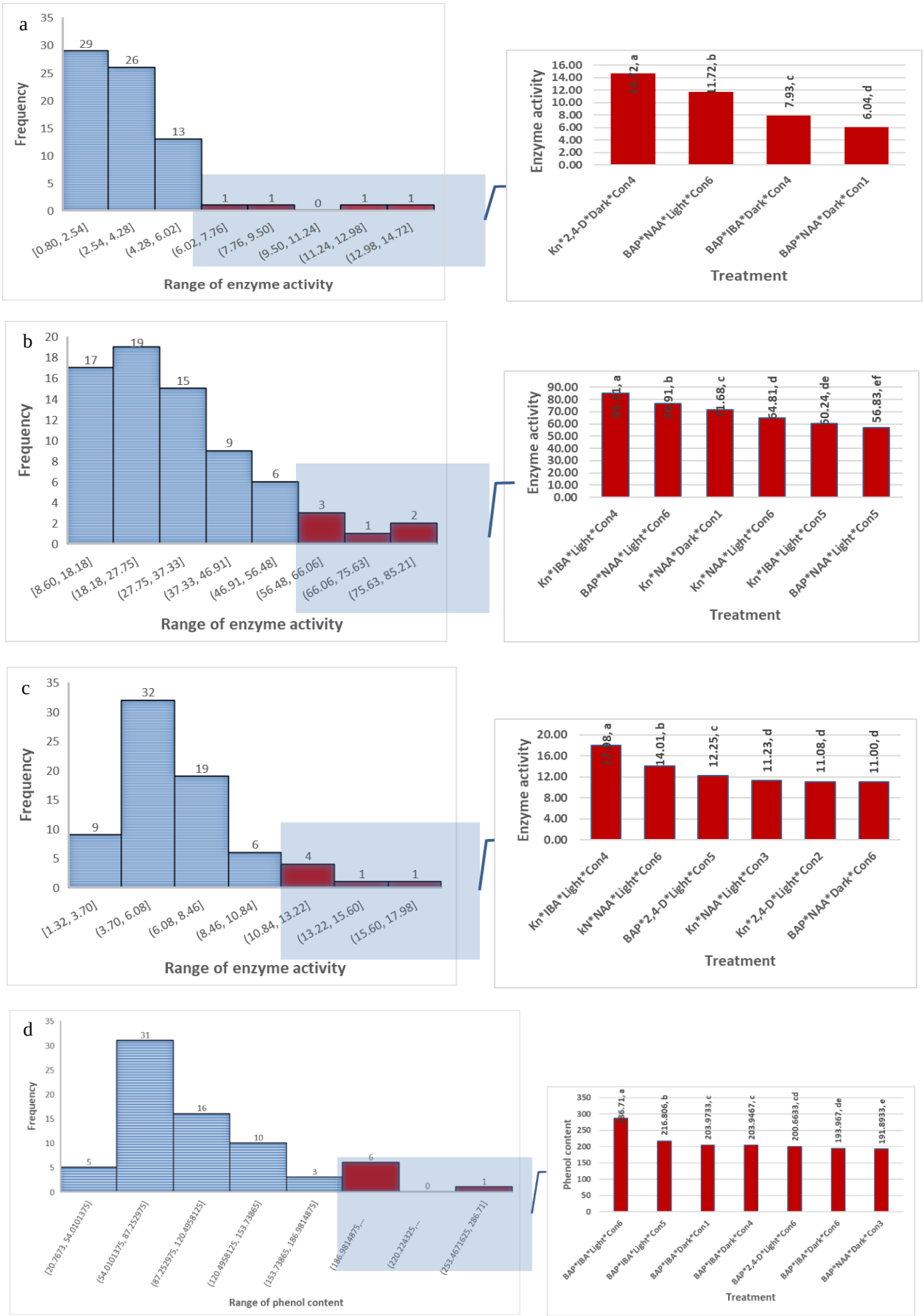

Fig. 2 Frequency distribution of enzyme activity or phenol content under three-way interactions of PGRs × hormone concentration × light (graphs on the left). Treatments leading to the highest activity of the catalase (a), peroxidase (b), superoxid dismutase (c) and Total phenol content (d) are provided in the right graphs. In each graph values followed by different letters are significantly different according to a Duncan's test at $P< 0.05$.

Phenolic components and antioxidant enzymes are the most important parts of the antioxidant system in plants. Enhancement of activities of antioxidant enzymes by PGRs application was reported in tissue culture and plants under both normal and stress conditions [40]. Our results illustrated that both the combination and the concentration of PGRs can exert influence on the total phenolic content and activity of antioxidant enzymes of callus. In *N. sativa*, treatment with KN resulted in the enhanced activity of CAT, POD and SOD during salinity stress. The highest SOD and POD activities were detected in KN treated plants exposed to NaCl, whereas the highest CAT activity was measured in unstressed KN treated plants [41]. In *Morinda citrifolia* adventitious root culture, the highest activity of CAT was obtained by adding 0.3 mg/L KN or 0.5 mg/L TDZ. The maximum activity of POD was also reported in 0.5 mg/LTDZ, while in the case of KN the best concentration was 0.1 mg/L [42]. The callus of *Cnidium officinal* grown in MS medium supplemented with 2,4-D (27.1 µM) exhibited the highest total phenolic contents [43]. Total phenol content of *Sericostoma pauciflorum* callus reached the maximum level in an MS medium containing 2,4-D and BA [44]. The combination of IAA and 2,4-D, led to the production of higher polyphenols by the callus of *Nerium odorum* rather than using individual growth hormones IAA and 2,4-D [31]. Light is the most important factor in plants. Light is necessary for the photosynthesis of the whole plant and affects the production of callus and its related traits. In this study, light altered enzymes activities and total phenolic contents. In *C. officinale* the callus culture grown under dark conditions showed higher CAT and POD activities than ones grown in light conditions. On the contrary, total phenolic contents were in the lower amount [37]. Cell cultures *of Ajuga bracteosa* under continuous dark exhibited higher activities of SOD and POD, but the lower activity of CAT enzyme. In addition, the total phenol content in the dark was higher than that in the light. These values were reversed by adding methyl jasmonate as an elicitor [45]. In the batch culture of *Tetraselmis gracilis* the CAT activity peaked in the dark phase inconsistent with a 2.6-fold increase in the SOD activity in the light phase [46]. Contrary to our results, the calli of *Pyrostegia venusta* induced in 2,4-D and BAP showed higher total phenolic contents in the presence of light [47].

Plant cell culture offers the opportunity to establish a sustainable and eco-friendly method to produce plant natural products. Furthermore, the production of large amounts of callus is essential for the study of secondary metabolites. To optimize growth in callus mass production, it is crucial to select plant growth regulators (PGRs) prior to application. In our present study, several media fortified with different combinations and concentrations of PGRs were applied to induce callus from *N. arvensis*. Moreover, the involvement of the oxidative protection system in *N. arvensis* callus was investigated. It was found that enzymatic antioxidant activities and total phenolic contents were significantly affected by PGRs and environmental conditions.

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Conflict of Interest

The authors declare no conflict of interest.

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