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PRODUCTION OF CALLUS BIOMASS AND ANTIOXIDANT SECONDARY METABOLITES IN BLACK CUMIN

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ABSTRACT

Black cumin (*Nigella sativa*) is a medicinally significant plant species, used traditionally against a variety of diseases. In this investigation, efficient methods for *in vitro* seed germination and callus formation were developed. Further, the antioxidant potential was determined in the regenerated tissues. The results showed highest germination frequency in the black cumin seeds when incubated on MS (Murashige and Skoog, 1962) medium supplemented with 1.0 mg/L Gibberellic acid (GA_3). Among the different pretreatment solutions, H_2O_2 favored highest germination frequency followed by KNO_3. Callus biomass formation was initiated in cotyledon explants, and highest callus induction frequency (88%) was observed on MS media supplemented with 4.0 mg/L Thidiazuron (TDZ) plus 4.0 mg/L α-naphthalene acetic acid (NAA). In callus cultures, growth kinetics was inspected to determine the impact of culture period on antioxidant potential in relation to biomass accumulation. Highest dry biomass (13.2 mg/l) was recorded on day 35 of the growth curve at TDZ+NAA (4.0 mg/L; each). Further, more phenolics (TPC: 1.48 mg) and flavanoids (TFC: 0.58) were detected in calli harvested on day 35. Moreover, the DPPH radical scavenging activity in the callus cultures of black cumin was observed in correlation with accumulation of dry biomass.

Key words: Black cumin, callus, growth curve, phenolics, antioxidant activity.

Abbreviations: PGR=Plant growth regulator, DBM=Dry bio mass, TPC= Total phenolic content, TFC= Total flavonoid content, FRSA=Free radical scavenging activity, MS= Murashige and Skoog, DPPH= 1,1-diphenyl-2-picryl-hydrazyl

INTRODUCTION

Black cumin (*Nigella sativa*) of family Ranunculaceae is cultivated globally for its black seeds which are used as a preservative or spice agent in a variety of food products (Atta 2003; Salem 2005). Seed oil extracted from wild grown black cumin has many applications in contemporary naturopathic therapeutics such as antidiarrheal, carminative, appetite stimulant, liver tonic and in treatment against asthma, respiratory oppression, bronchospasm coughs, back pain, obesity and hypertension (Abdel-Zaher *et al*. 2011). Despite its enormous biological potency, the wild grown black cumin plants are exhibiting extreme variability in the phytochemical profiles due to adverse effects of fluctuating climatic and environmental conditions. Further, lack of local and uniform farming practices has restricted the production of phytochemically consistent black cumin plantlets, those can be used in preparation of effective phyto medicines (Khan *et al*. 2015). Using the biotechnological methods for instance *in vitro* seed germination, micropropagation and callus organogenesis can prevent the issues of variability and thus can provide promising means for production of healthy plant material, irrespective of the environmental constrains in controlled conditions and in limited space (Abbasi *et al*. 2010; Khan *et al*. 2013). *In vitro* seed germination is a good strategy for production of plants with consistent phytochemical profiles under controlled and aseptic conditions in a short span of time (Khan *et al*. 2013; Nikolic *et al*. 2006). Besides, this technique provides continuous source of different explants which can be used as a starting material for establishment of different *in vitro* cultures in the time when the natural growing season is off (Shoji *et al*. 2008; Khan *et al*. 2013). Callus cultures can serve the *in vitro* production of a variety of bioactive compounds. Besides callus can also be used as a type of explant for establishment of somatic embryogenesis, rhizogenesis or can be converted into shoots, depending on the type of growth regulators and culture media (Khan *et al*. 2014). The *in vitro* regenerated plant tissues produce comparatively higher amount of phenolic, flavonoid and antioxidant components than wild counterparts (Khan *et al*. 2014). Usually during unusual conditions, different reactive oxygen species (ROS) are produced which interact with biological molecules and cause multiple problems such as inhibition of growth, differentiation and metabolite production (Shoji *et al*. 2008). When produced in higher levels, the ROS can induce cell toxicity, resulting in number of human diseases including inflammation, coronary disorders, carcinoma, neuron-degeneration and cancer (Dreher and Junod, 1996;

Halliwell, 2001; Halliwell and Gutteridge, 2007). Plant cell copes with the detrimental effects of ROS through action of its enzymatic system via functional antioxidative enzymes or through non-enzyme components like plant poly phenols (Valko *et al*. 2006; Khan *et al*. 2017). Within the different classes of phytochemicals, phenolics and flavonoids are known to have higher antioxidant potential as compared to carotenoids and vitamins (Cie´sla *et al*. 2012). Looking into the medicinal significance of antioxidant metabolites, black cumin was exploited *in vitro* for incrementing the antioxidant activity. The current investigation was aimed to develop feasible methods for *in vitro* seed germination and callus formation in black cumin. Moreover TPC, TFC and DPPH^o antioxidant activity were also determined in the regenerated callus tissues.

MATERIALS AND METHODS

Plant germplasm and sterilization: Black cumin seeds were obtained from National Agriculture Research Centre (NARC) Islamabad, Pakistan in 2014. Viability of seeds was confirmed by using float test method. Surface sterilization of the viable seeds was employed as per the established protocol of Abbasi *et al*. (2010). Briefly, seeds were treated with 70% (v/v) ethyl alcohol for 3 min followed by washing step with distilled water, further treated with 0.1% (w/v) $HgCl_2$ for 5 min and finally washed 3 times with autoclaved distilled water.

***In vitro* seed germination and explants preparation:** In preliminary studies, seeds were germinated in Petri dishes lined with filter paper under the laboratory condition (22-25 ^{0}C). Filter paper was moistened by distilled water after every 3 days. In another set of experiments, seeds were employed *in vitro* on MS medium with or without addition of Gibberellic acid (GA_3) at varying levels (0.1 – 2.0 mg/L). For further germination tests, seeds were treated with KNO_3 (1%) (v/v), H_2O_2 (0.5 %), H_2SO_4 (0.5 %) and boric acid (1%) as pretreatment solutions.

Callus induction, biomass formation and growth dynamics: Initially four different explants were tested on MS medium containing 4.0 mg/L NAA for callus induction. Due to its higher morphogenetic potential, cotyledon explants were selected for studying callus growth kinetics. Cotyledon explants (~2.5 mm^2) were taken from one month old *in vitro* germinated black cumin plantlets and were subsequently cultured on MS medium containing 3% sucrose (w/v), 0.8% agar (w/v) and varying levels of different plant growth regulators (PGRs). Within the different PGRs, notable cytokinins including kinetin (Kn), benzyl-aminopurine (BAP) and thidiazuron (TDZ) and auxin such as α-naphthalene acetic acid (NAA) were tested *in vitro* at concentrations (2.0-6.0 mg/L) either individually or combination of the cytokinins with 4.0 mg/L NAA. For control treatment, MS zero medium i.e media containing no PGRs was used. The growth chamber was adjusted with temperature at 25 ± 2ºC and lighting conditions for control photoperiod (16 hr. light & 8 hr. dark). Data on several aspects of callus growth was recorded as (i) frequency of callus induction in explants (ii) diameter of callus and (iii) morphological characteristics of callus cultures after one month of culture cultivation. For growth kinetics 30 days old callus was harvested and 0.1 g callus tissue was inoculated on MS media combined with 4.0 mg/l TDZ plus 4.0 mg/l NAA. Bio mass formation in callus cultures was measured as fresh weight (FW) and dry weight (DW) after every 7 days in total of 42 days. All experiments were performed in triplicate culture flasks and were repeated twice.

Phytochemical estimation of the callus cultures: Extraction of the metabolites from the samples was carried according to the modified protocol of Khan *et al*. (2013). For the determination of total phenolic content (TPC) and total flavonoid content (TFC) in the regenerated plant samples, the methods of Velioglu *et al*. (1998) and Chang *et al*. (2002) were used respectively. Antioxidant activity in the *in vitro* raised samples was demonstrated according to the protocol of Abbasi *et al*. (2010). Briefly, 20 µl of each biological sample were mixed with 180µl of DPPH^o solution in each well of 96 well microplate. The mixture was kept in dark for 1hour at 25 ºC. The absorbance was measured at 517 nm. The free radical scavenging activity (FRSA %) was formulated as:

% scavenging DPPH free radical = 100 x (1-AE/AD)

Where, AE denotes absorbance of the mixture at 517 nm and AD denotes the absorbance of the DPPH solution without adding anything.

Statistical analysis: Data was collected from all the experiments in triplicates and subjected to statistical analysis by using Statistix 8.1. Statistical significance in data was determined by one way ANOVA at $P < 0$. Data figures were generated through Origin 8.5.

RESULTS AND DISCUSSION

***In vitro* seed germination:** The confined seed dormancy is the major constrain in cultivation of medicinal and aromatic plants. For successful micropropagation protocol it is vital to have an aseptic continuous supply of germplasm as a source of explants. Thus *in vitro* seed germination can be a promising source of hygienic germplasm for preparation of different explants (Khan *et al*. 2013). In this study, highest seed germination frequency (91%) was observed when surface sterilized black seeds were incubated on MS medium containing 1.0 mg/l GA_3 (Fig. 1a). An increase in level of GA_3 beyond an optimal level resulted in decline of

germination frequency. GA_3 is reported for its profound activity to overcome dormancy in *Parthenium argentatum* (Dissanayake *et al*. 2010). Among the germination tests with different chemicals, Hydrogen peroxide induced higher germination frequency in seeds followed by KNO_3; however, the lowest germination frequency (33%) was observed in seeds on filter paper moisten with distilled water. Black cumin seeds usually possess dormancy and even in presence of favorable conditions, seeds fail to germinate in their natural habitat. However there are many other factors too which can inhibit seed germination (Finch S & Leubner M, 2006). Preconditioning treatments of seeds with various chemical agents have proven as effective strategies for breaking the physiologically confined seed dormancy and enhancing germination frequency in recalcitrant plant species (Shah, 2007). Similar to our results GA_3 and KNO_3 have shown significantly positive effects on germination in *Aconitum heterophyllum* (Srivastava *et al.* 2008). However, preconditioning treatments of seeds, such as application of H_2O_2 and KNO_3 may not necessarily enhance seed germination in many plant species (Jorge *et al.,* 2006).

Effects of explants type and PGR on callus induction frequency: Several types of explant were exploited in preliminary experiments for determining the most pertinent explant in callus organogenesis (Fig. 1b). However, cotyledon explants showed highest potential of callus formation (54%) on MS medium supplemented with 4.0 mg/L NAA and was exploited for subsequent extensive experiments with different other PGRs (Table 1).

Callus induction frequency was incremented further (88%), when cotyledon explants were incubated on MS medium containing NAA and TDZ at 4.0 mg/L each. After seven days of explants inoculation on the culture media, the cut ends of the explants were observed for initiation of callus formation.

Callus growth characteristics were observed visually and were found significantly different in response to different PGRs tested (Table 1). Soft, friable and yellowish green calli with diameter (1.3 cm) were observed in culture flasks at the aforementioned PGR treatment after four weeks of explant cultivation. Similar observations in callus formation were observed in *Silybum marianum* by Khan *et al.* (2014). Higher levels of TDZ favored higher callus formation in current study, however a decline in callus organogenesis was observed for Kin or BAP at higher levels when combined with 4.0 mg/L NAA.

Thidiazuron has been reported as the potent growth regulator in callus induction and organogenesis in many commercially important plant species. The higher morphogenic potential of TDZ can be explained by its regulatory role in acquisition of the explants cellular competency to activate the metabolic pathways, resulting in the production of the elevated levels of the indigenous growth hormones, particularly cytokinins those in turn can facilitate induction and proliferation of callus (Khan *et al*., 2014).

Lowest callus formation frequency (30%) was observed on MS medium supplemented with 2.0 mg/L BAP. However the callus induction frequency was enhanced by combination of 2.0 mg/L of BAP with 4.0 mg/L of NAA. No callus induction response was observed in control treatment (MS0).

In another study, highest callus induction frequency (82%) of *N. sativa* was observed when epicotyle explants were cultured on MS medium containing 2.0 mg/L Kin +1.0 mg/L NAA (Chaudhry *et al*., 2014). Alemi *et al*. (2013) reported highest callus induction in *N. sativa* explants on MS medium containing 1.0 mg/L 2,4-D plus 2.15 mg/L Kn. In accordance to our data, the application of NAA alone or in combination with BAP enhanced callus formation in *Silybum marianum* (Khan *et al*. 2014). During explants culturing negligible (2%) microbial contamination was observed. This might be due to the fact that the explants used were taken from *in vitro* grown seedlings which are often less susceptible to surface contamination in comparison to *in*

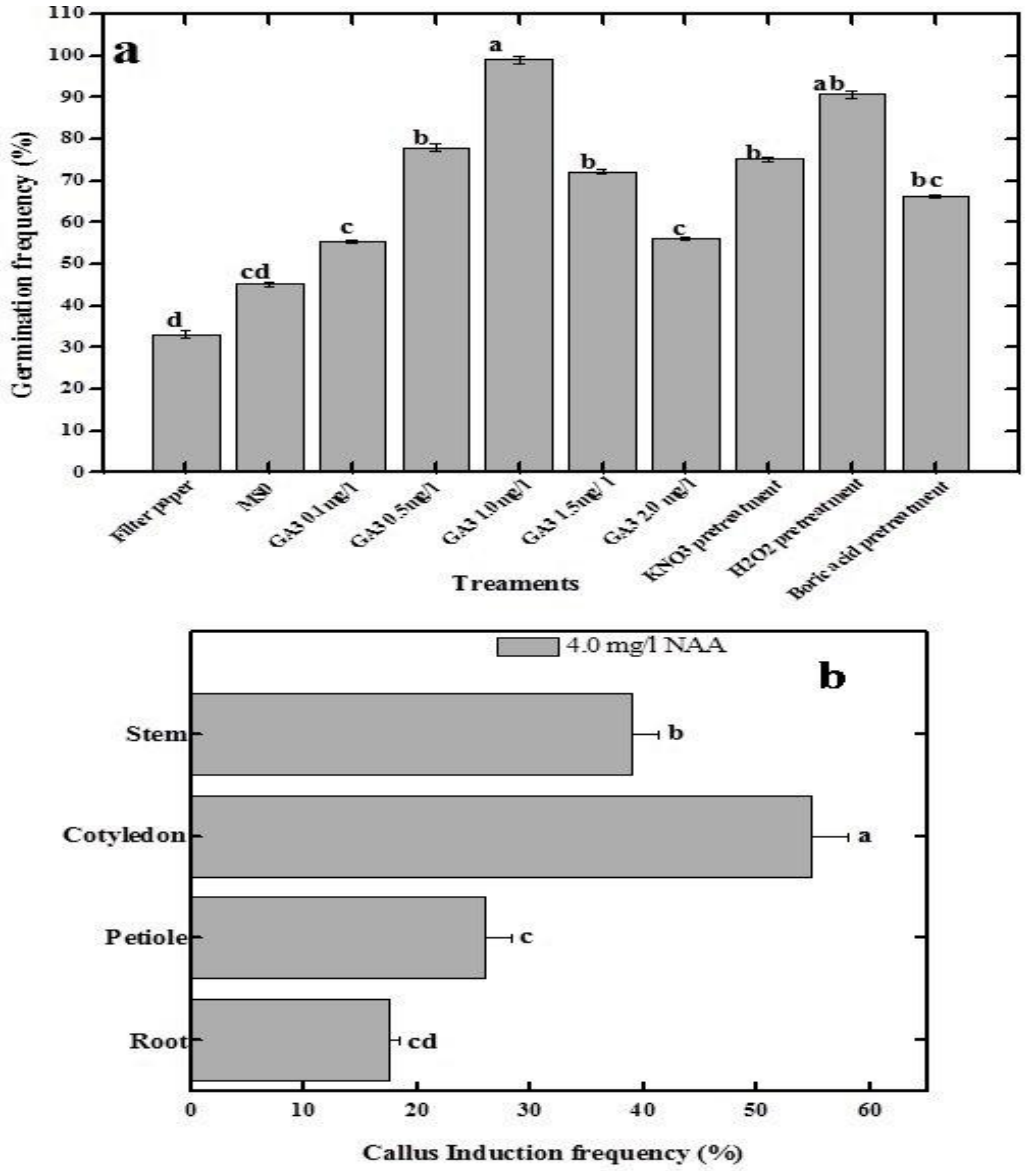

Figure 1:

a): Assessment of the effects of pre-soaking treatments with different chemicals on seed germination frequency in *Nigella sativa* L. Data are the mean values of triplicate experiments with ± SE. Different alphabets on the columns represent significance in data at $P < 0$.

b): Assessment of the effects of different explants on *in vitro* callus induction frequency on MS medium supplemented with (4.0 mg/L) NAA. Data are the mean values of triplicate experiments with ± SE. Different alphabets on the columns represent significance in data at $P < 0$.

vivo raised explants for micropropagation (Abbasi *et al.,* 2010). Callus organogenesis is attributable to multiple factors for instance type of explant, genetic makeup of the explant, type of PGR, nutrient media and *in vitro* culture conditions (Khan *et al*. 2014)

Callus growth kinetics: In callus cultures, growth kinetics was inspected to determine the impact of bioprocessing on antioxidant potential in relation to biomass accumulation. Growth curve was characterized by distinct growth stages of lag phase (7 days), log phase (28 days) and stationary phase (7 days) for accumulation of biomass (Fig. 2). Compared to starting inoculum, more than eight-fold increase in biomass was observed. Within the log phase, on day 35 maximum biomass accumulation (FBM: 155 mg/l, DBM: 13.2 mg/l) was harvested in the growth curve. Color of callus culture was also changed during growth kinetics. It was light green at lag phase, yellowish green during log phase and brownish in the stationary phase. Further, a direct correlation was

observed between callus dry biomass accumulated with days in the growth curve. In a similar study in *Artimisia absinthium*, Ali *et al*., (2013), reported highest biomass accumulation on MS medium fortified with TDZ and NAA at 1.0 mg/L each.

Biochemical parameters during growth kinetics of callus culture: Plant polyphenols are the bioactive secondary metabolites, produced as a consequence of stress condition by plant cell to mitigate the detrimental effects of stress mediated free radicals. The important classes of the polyphenols are the low molecular weight phenolic acids and flavonoids, which are having prominent antioxidant activity and are of pharmacological interest in treatment of many human diseases (Khan *et al*. 2015). The higher amount of phenolics (1.48 GAE-mg/g-DW) and flavonoids (0.58 RE-mg/g-DW) were detected on day 35 in the callus growth curve (Fig.4a&b). Both TPC and TFC displayed a growth dependent pattern and were found in correlation with DBM (r=0.96, p=0.095) in the growth curve (Table. 2). During unusual conditions, reactive oxygen species (ROS) such as $O2^-$ are produced in abundant amount that can cause an oxidative burst and eventually damage the cells (Sreelatha and Padma, 2009). Phenolics and flavonoids are produced as defense chemicals in plants and act as efficient scavengers of the ROS (Kosar *et al*.2011). Assessment of the antioxidant potential through $DPPH^0$ free radical scavenging method can confirm the antioxidant nature of the metabolites present in a plant sample (Abbasi *et al*. 2010). Highest antioxidant activity (88%) was observed on day 42 in the growth curve (Fig. 4c). Further, a sequential increase in activity was observed with progression of days during growth kinetics. Interestingly, the DPPH free radical scavenging activity correlated the DBM (r= 0.96, p= 0.023) in the growth curve (Table. 2). The antioxidant potential of natural products is due to the presence of phenolics and flavonoids present in a variety of medicinal and aromatic plants (Matkowski, 2006). Therefore, plant *in vitro* technologies are widely exploited for the production and enhancement of such high value added natural products as promising source of natural antioxidants (Abouzid *et al*. 2010).

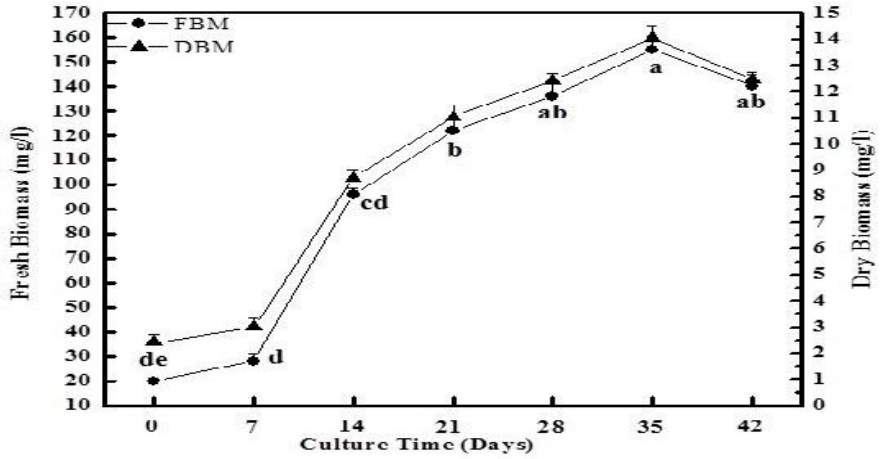

Figure 2: Growth kinetics and biomass accumulation of callus culture in *Nigella sativa* L on MS medium supplemented with TDZ (4.0 mg/L) +NAA (4.0 mg/L). Data are the mean values of triplicate experiments with ± SE.

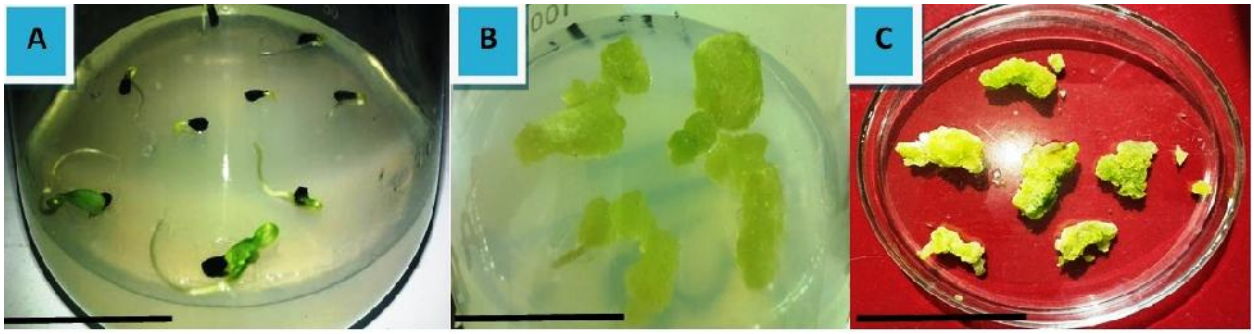

Figure 3: Pictorial presentation of the *in vitro* cultures in *N. sativa*. A): *In vitro* seed germination B and C): Callus formation, bar=1mm

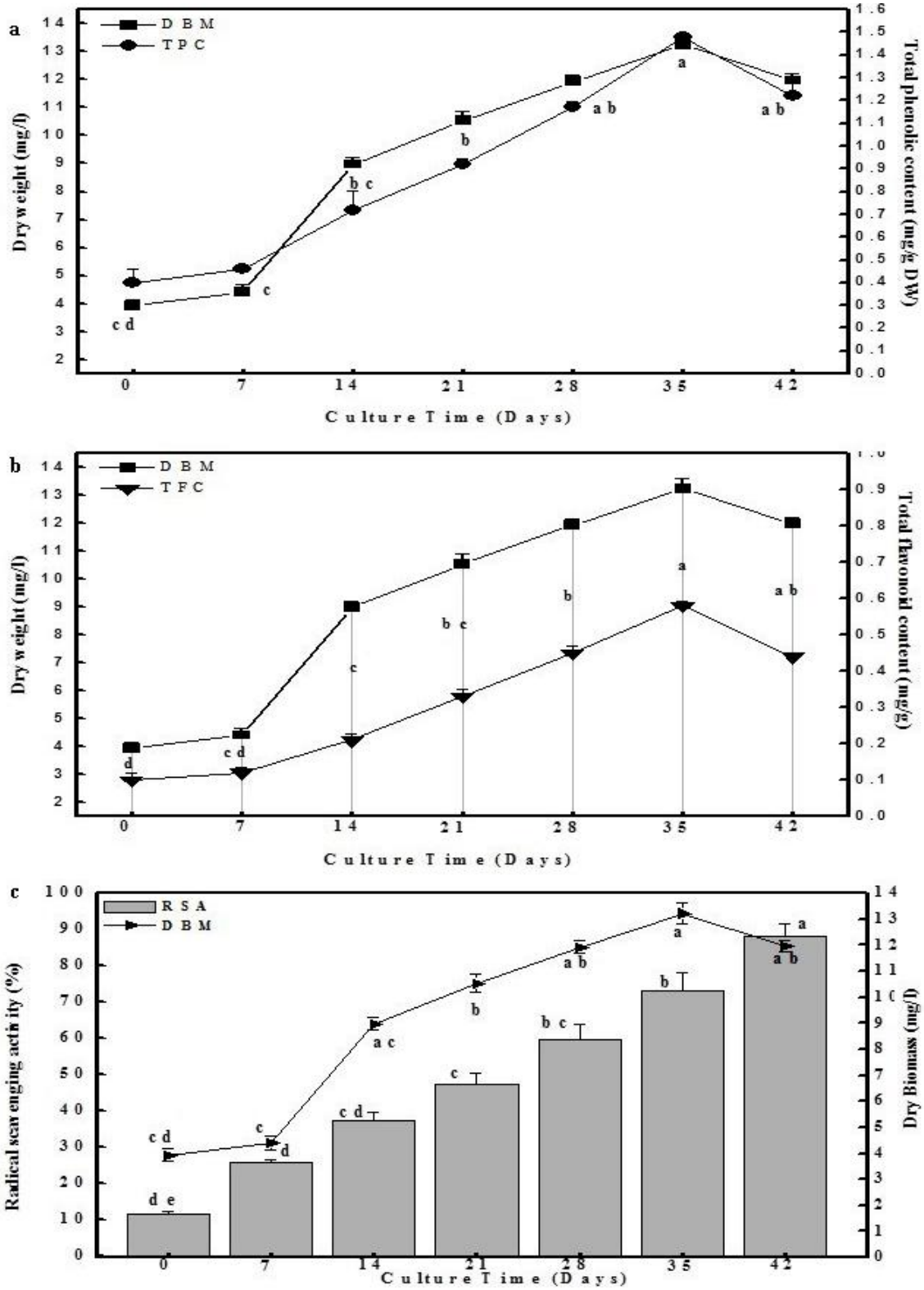

Figure 4: Antioxidant potential in relation to biomass accumulation of callus culture in *Nigella sativa* L on MS medium supplemented with TDZ (4.0 mg/L) +NAA (4.0 mg/L). (a): TPC (b): TFC and (c): antioxidant activity (%).

Table 1. Effects of varying levels and combinations of auxins or cytokinins on callus induction and growth characteristics in *Nigella sativa* L.

PGRs (mg/L)	Callus induction frequency	Callus diameter (cm)	Callus color	Callus texture
MS0	---	---	---	---
2 NAA	48.9±2.33^{bc}	1±0.15^{b}	Yellowish green	Friable
4 NAA	56.5±1.94^{bc}	1.2±0.08^{ab}	Yellowish green	Friable
6 NAA	34.8±2.25^{cd}	0.9±0.01^{b}	Yellowish green	Friable
2 BA	30.7±2.48^{cd}	0.5±0.01^{cd}	Dark green	Hard
4 BA	55.7±2.65^{bc}	0.7±0.02^{bc}	Dark green	Hard
6 BA	48.6±2.79^{c}	0.3±0.02^{cd}	Dark green	Hard
2 Kn	43.7±1.30^{c}	0.2±0.01^{d}	Light green	Granular
4 Kn	59.6±1.59^{b}	0.4±0.03^{cd}	Light green	Granular
6 Kn	33.9±1.87^{cd}	0.3±0.02^{cd}	Light green	Granular
2 TDZ	54.6±2.39^{bc}	0.5±0.03^{c}	Yellowish green	Friable
4 TDZ	77.3±2.81^{ab}	0.8±0.02^{bc}	Yellowish green	Friable
6 TDZ	67.2±2.94^{b}	0.6±0.02^{c}	Yellowish green	Friable
2 KN +4 NAA	61.9±3.37^{b}	0.4±0.03^{cd}	Light green	Soft & loose
4 KN +4 NAA	72.3±3.24^{ab}	0.6±0.01^{c}	Light green	Soft & loose
6 KN +4 NAA	35.8±2.68^{cd}	0.3±0.03^{cd}	Light green	Soft & loose
2 BA +4 NAA	64.9±3.16^{b}	1.3±0.01^{a}	Dark green	Granular
4 BA +4 NAA	57.7±2.83^{b}	1.1±0.02^{ab}	Dark green	Granular
6 BA +4 NAA	33.1±1.88^{cd}	0.9±0.02^{b}	Dark green	Granular
2 TDZ +4NAA	68.6±3.82^{ab}	0.8±0.02^{bc}	Yellowish green	Friable
4 TDZ +4NAA	88.6±3.69^{a}	1.5±0.11^{a}	Yellowish green	Friable
6 TDZ +4NAA	72.1±3.91^{ab}	1.2±0.01^{ab}	Yellowish green	Friable

Table 2. Descriptive statistics of the impact of the cultivation period during growth kinetics on antioxidant potential in relation to biomass accumulation of callus culture in *Nigella sativa* L. (Min= Minimum, Max= Maximum, SEM= Standard Error of Mean

	Mean	Variance	SEM
DBM	9.28	13.98	1.41
TPC	0.91	0.16	0.15
TFC	0.32	0.03	0.07
FRSA	48.86	721.55	10.15

Conclusion: It is concluded that cotyledon explants incubated on MS medium containing 4.0 mg/L TDZ plus 4.0 mg/L NAA can produce abundant amount of callus. Bioprocessing enhanced further the biomass formation and antioxidant activity in callus cultures. A biomass dependent pattern in the antioxidant secondary products was observed in the growth curve. Thus a simple and efficient method for production of biomass and antioxidant potential in callus cultures of Black cumin was established, which has the potential for industrial production of health promoting natural products.

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Author contribution: Research work presented in this manuscript was carried out in collaboration between all authors. ZRM defined the research theme and planned the experiments. AB and MA performed the experimental work. MAK and MA evaluated the results and wrote the manuscript.

Competing interest: All the authors declare no conflict of interest.

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