Exogenous melatonin trigger biomass accumulation and production of stress enzymes during callogenesis in medicinally important *Prunella vulgaris* L. (Selfheal)

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Received: 26 September 2017 / Revised: 6 March 2018 / Accepted: 5 June 2018 / Published online: 18 June 2018
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Abstract The objective of the current study was to monitor the variations caused by the application of exogenous melatonin on growth kinetics and production of stress enzymes in *Prunella vulgaris*. Leaf and petiole explants were used for callogenesis. These explants were inoculated on Murashige and Skoog media containing various concentrations of melatonin alone or in combination with 2.0 mg/l naphthalene acetic acid. Herein, a maximum of 3.18-g/100 ml fresh biomass accumulation was observed on day 35 during log phase of growth kinetics at 1.0 mg/l melatonin concentration from leaf explants. While 0.5 and 1.0 mg/l melatonin enhanced the biomass accumulation from petiole explants. Moreover, the synergistic combination of melatonin and naphthalene acetic acid also promoted growth from leaf and petiole explants. Leaf derived callus cultures treated with 1.0 mg/l melatonin induced the production of total protein content (90.47 µg BSAE/mg FW) and protease activity (4.77 U/g FW). While the calli obtained from petiole explants have shown highest

content of total protein (160.8 µg BSAE/mg FW) and protease activity (5.35 U/g FW) on media containing 0.5 mg/l melatonin. Similarly, 0.5 mg/l melatonin enhanced superoxide dismutase (3.011 nM/min/mg FW) and peroxidase (1.73 nM/min/mg FW) enzymes from leaf derived callus cultures. The combination of 1.0 and 1.5 mg/l naphthalene acetic acid enhanced content of total protein and protease activity in leaf and petiole derived cultures. These results suggested that the application of melatonin play a positive role in biomass accumulation and production of stress enzymes in *P. vulgaris*.

Keywords *P. vulgaris* · Melatonin · Callogenesis · Growth kinetics · Stress enzymes

Introduction

Plants are exposed to a variety of environmental factors that include extreme low and high temperatures, pH variation (alkalinity, acidity), light intensity, drought and damages due to oxidative stress. Morphological, physiological, biochemical and molecular changes are triggered in plants as a result of these environmental changes (Ahmad et al. 2012). A number of factors like temperature; light, pH, aeration and agitation affecting the production of metabolites have been studied extensively (Treju-Espino et al. 2011; Nagella and Murthy 2010). Like physical factors, chemical factors including phytohormones, media composition and various other elicitors also affected the production of secondary metabolites (Treju-Espino et al. 2011; Lee and Shuler 2000). By optimizing the cultural conditions, several products accumulated at an elevated level in cultured cells than in intact plants (Baque et al. 2012; Matsubara et al. 1989; Karwasara and Dixit 2012).

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Manipulation of nutritional components as well as physical factors received by the culture is the primary approach for optimizing the culture productivity.

Melatonin, a mammalian indoleamine neuro-hormone, synthesized in the pineal gland of mammals is regarded as a ubiquitous and highly conserved molecule. It mediated the stress caused by chemical and environmental factors (Reiter et al. 1997; Zhao et al. 2011a, b). More than 5700 reports are available on the occurrence of melatonin in various plants and animal species. Relatively high levels of melatonin are present in the leaves and flowers of several medicinal plants and is used to treat neurological disorders (Murch et al. 2001). Relative ratios of melatonin and serotonin regulated the light/dark responses, circadian rhythms and seasonality and also modulated the in vitro morphogenesis in plants (Kolar et al. 1997; Murch and Saxena 2002). Furthermore, melatonin is reported to control physiological processes including diurnal responses, environmental adaptations and detoxification of free radicals. Murch et al. [9] reported the potential of melatonin as a plant growth regulator that modulated the de novo organogenesis in *Hypericum perforatum*. Sheshadri et al. (2018) recently studied that melatonin has been involved in the expression of reference genes in *Catharanthus roseus*. Shi et al. (2017) documented that melatonin is an important pleiotropic agent with multiple cellular responses in animals and plants. Wang et al. (2018) suggested that melatonin enhance plant tolerance to salt, drought, heat and cold. Nawaz et al. (2018) further validated that melatonin regulate antioxidant gene expression and improve vanadium stress tolerance of watermelon seedlings.

P. vulgaris of the family Lamiaceae, is commonly known as self-heal and is used for many ailments in various systems of medicines (Liu et al. 2009; Shinwari et al. 2006). It has a range of biological effects including antispasmodic, anti-cancerous, antiseptic, antirheumatic and against human immunodeficiency virus and herpes simplex virus (Huang et al. 2013; Rasool et al. 2009). There is a high demand for production of the medicinal plants such as *P. vulgaris* due to their remedial properties, leading to an increased burden on natural repositories of plants. Unfortunately, lesser information is available about production of *P. vulgaris* both in field and in vitro conditions. According to Ali et al. (2013), the development of callus cultures minimized the time required for the production of complete plantlets and secondary metabolites and has overcome the limitation of its low quantities generated in parental plants along with overcoming the difficulty of synthesizing under laboratory conditions.

Keeping in view these considerations, the influence of melatonin as elicitor was investigated in *P. vulgaris* for in vitro callus development and production of content of

total protein, superoxide dismutase, peroxidase enzymes and determination of protease activities.

Materials and methods

Establishment of callus cultures from leaf and petiole explants

To develop callus cultures, leaf and petiole explants were obtained from in vitro seed derived plantlets of *P. vulgaris*. In previous studies, 2.0 mg/l of naphthalene acetic acid was found to be the best plant growth regulator for callus induction from leaf and petiole explants (Fazal et al. 2016a, b, c, d). Therefore, approximately, 3–4 mm² leaf pieces were placed on Murashige and Skoog (1962) supplemented with 2.0 mg/l of naphthalene acetic acid. The Murashige and Skoog media was augmented with 30 g l⁻¹ sucrose, solidified with 8 g l⁻¹ agar (Agar Technical LP0013, Oxoid, Hampshire, England), the pH was adjusted to 5.3 (Eutech Instruments pH 510, Singapore) and all the media were autoclaved (Systec VX 100, Germany) at 121 °C for 20 min. All cultures were maintained in a growth room at temperature of 25 ± 1 °C under a 16/8 h photoperiod with a light intensity ranging from ~ 40 to 50 μmol m⁻² s⁻¹ provided by fluorescent tube lights (20 W, Toshiba FL20T9D/19; 380–780 nm). Murashige and Skoog medium with 2.0 mg/l of naphthalene acetic acid was also used as control with same experimental conditions.

Determination of growth kinetics under the influence of melatonin treatments

To study the effects of melatonin on callus proliferation, Murashige and Skoog basal media augmented with different concentrations of melatonin alone (0.5, 1.0, 1.5 and 2.0 mg/l) or in combination with naphthalene acetic acid (2.0 mg/l) were applied. Data regarding growth kinetics was collected with 7 days interval for a period of 49 days. Growth curve was established for the accumulated biomass of the rapidly growing calli in response to different concentrations of melatonin alone (0.5, 1.0, 1.5 and 2.0 mg/l) or in combination with naphthalene acetic acid (2.0 mg/l). For fresh weight (FW) determination, calli were collected from solid Murashige and Skoog media, carefully washed with sterile distilled water, pressed gently on filter paper (Whatman Ltd., England) to remove excess water and finally weighed (Sartorius digital balance; Germany). Similarly, for dry weight (DW) investigation, calli were dried in an oven (Thermo Scientific; Germany) at 50 °C and finally weighed. Fresh and dry weights were expressed in gram/100 ml.

Estimation of antioxidative enzyme activities

To quantify set of antioxidative enzymes, calli cultures were extracted according to the protocol of Nayyar and Gupta (2006) with little modifications. Practically, 1 g fresh sample was taken in a test tube and thoroughly mixed with 10 ml of extraction buffer (50 mM KH_2PO_4 buffer with 1% polyvinylpyrrolidone at pH 7). The homogenized mixture was then centrifuged (14,000 rpm) at 4 °C for 30 min. The supernatant was collected in sterile tube and later on applied for enzyme assays. For the determination of peroxidase activity, the method of Lagrimini (1991) was followed with some modifications. Superoxide dismutase activity was investigated by using the method of Ahmad et al. (2014).

Determination of total protein content and protease activity

The protocol of Giri et al. (2012) was used for calli extraction with some modification. Practically, 4 g fresh sample was pulverized in a chilled pestle and mortar with liquid nitrogen and 200 mg powder was mixed with 1 ml of methanol and subsequently placed in a cold room for incubation (5 min). The sample was then exposed to sonication (5 min; Toshiba, Japan), vortexing (20 min) and centrifugation (13,000 rpm, 5 min). The supernatant was used for analysis and a regression curve of the standard solutions of various concentrations was worked out against their respective absorbance. For determination of total protein content, the method of Lowry et al. (1951) was followed. The absorbance of the supernatant was measured at 650 nm using a spectrophotometer (Shimadzu, ultra-violet-120-01) and standard curve of bovine serum albumin was then prepared by absorbance versus micrograms protein or vice versa and the unknown protein from the sample was determined from the curve. Further, the protocol of McDonald and Chen (1965) was followed for determination of protease activity.

Data analysis

All experiments were repeated twice and each treatment consisted of three replicates. Mean values of various treatments were subjected to analysis of variance and for standard errors ($\pm$) and least significant difference Statistix software (8.1 versions) was used. For graphical presentation, Origin Lab (8.5) software was used.

Results and discussion

Melatonin induced biomass accumulation from leaf and petiole explants in callus cultures of *P. vulgaris*

In this experiment, calli proliferations from leaf and petiole explants were investigated for biomass accumulation under the influence of melatonin alone (0.5–2.0 mg/l) or in combination with naphthalene acetic acid (2.0 mg/l) as shown in Fig. 1. An average leaf explants (0.0114 g) were inoculated on media containing different concentrations of melatonin. During growth kinetics, most of the cultures showed lag phase from 7 to 21 days (Fig. 2a). After 21 days of incubation, most of the cultures showed log phase up to 35 days. The decline phase was observed from 36 to 49 days. During the log phase, most of the cultures accumulated maximum biomass. Comparatively, 1.0 mg/l melatonin displayed maximum biomass in lag (1.16, 1.38 and 1.42 g/100 ml), log (2.29 and 3.18 g/100 ml) and decline phases (1.61 and 1.35 g/100 ml) as compared to other concentrations and control. In contrast, calli obtained from petiole explants showed higher accumulation of biomass during different growth phases on media containing 0.5 and 1.0 mg/l melatonin (Fig. 2b). The log phase was observed up to 35 days of inoculation followed by the decline phase. These results showed that lower concentrations of melatonin were found effective for biomass accumulation during growth curve. Lower concentrations of melatonin have a stimulatory effect on root growth of *Brassica juncea*, whereas higher concentration has an inhibitory effect on it (Chen et al. 2009). Melatonin stimulates the expansion of etiolated cotyledons, which have a direct relationship with biomass accumulation in *Lupinus albus* L. (Hernandez-Ruiz and Arnao 2008). The current data was also supported by the results of Afreen et al. (2006) that dose-dependent manner of melatonin promoted the vegetative growth and development of *Glycyrrhiza uralensis*. Melatonin is reported to influence the development of stems and leaves, promotes rhizogenesis and calllogenesis in explant cultures and cryopreserved callus or shoot tips for long-term storage in various medicinal herbs (Zhao et al. 2011a, b; Murch and Saxena 2002; Murch et al. 2001; Jones et al. 2007; Sarropoulou et al. 2012; Zhang et al. 2013; Szafranska et al. 2013; Uchendu et al. 2013).

Melatonin and naphthalene acetic acid induced biomass accumulation from leaf and petiole explants in callus cultures of *P. vulgaris*

Leaf and petiole explants were inoculated on Murashige and Skoog media containing various combinations and concentrations of melatonin and naphthalene acetic acid.

Fig. 1 Effects of melatonin and naphthalene acetic acid on callus growth in *P. vulgaris* **a** callus induction from petiole explant on medium containing 2.0 mg/l naphthalene acetic acid, **b** callus induction from leaf explant on medium containing 2.0 mg/l naphthalene acetic acid, **c** 0.5 mg/l melatonin, **d** 1.0 mg/l melatonin, **e** 1.5 mg/l melatonin, **f** 1.5 mg/l melatonin, **g** 0.5 mg/l melatonin and 2.0 mg/l naphthalene acetic acid, **h** 0.5 mg/l melatonin and 2.0 mg/l naphthalene acetic acid, **i** 1.0 mg/l melatonin and 2.0 mg/l naphthalene acetic acid and **j** 2.0 mg/l melatonin and 2.0 mg/l naphthalene acetic acid

Fig. 2 Effects of melatonin on biomass accumulation during callogenesis in *P. vulgaris* **a** growth kinetics of biomass accumulation from leaf explants, **b** biomass accumulation from petiole explants, **c** effect of synergistic combination of melatonin and naphthalene acetic acid on biomass accumulation from leaf explants and

d combined effect of naphthalene acetic acid and melatonin on biomass accumulation from petiole explants. Data was collected from 3 independent experiments. Mean values with standard errors ($n = 3$) are significantly different at $P < 0.05$

The growth curve was established for accumulated biomass for 49 days period (7 weeks). During growth kinetics, maximum biomass accumulation (3.89 g/100 ml) was observed on media containing 1.5 mg/l melatonin and naphthalene acetic acid from leaf explants on day 35 of log phase (Fig. 2c). Almost all the cultures showed a lag phase of 21 days, log phase of 2–3 weeks and decline phase of 2 weeks (36–49 days). However, 0.5 mg/l melatonin and naphthalene acetic acid also induced significantly similar biomass accumulation from leaf explants as compared to control (1.27 g/100 ml). In contrast, combination of melatonin (0.5 mg/l) and naphthalene acetic acid (2.0 mg/l) enhanced the accumulation of biomass (5.22 g/100 ml) from petiole explants on day 35 during log phase of growth kinetics (Fig. 2d). It means that the combination of auxin

(naphthalene acetic acid) and melatonin enhanced biomass accumulation in slightly higher quantities than melatonin alone. Chen et al. (2009) suggested that low levels of melatonin enhanced the endogenous indole acetic acid biosynthesis, which stimulated in vitro growth, although the specific relationship between indole acetic acid and melatonin is still unclear. In the literature cited, the role of exogenous melatonin alone or combination of melatonin with other plant growth regulators on biomass accumulation in *P. vulgaris* is still scarce, however, few reports are available that melatonin enhanced the birefringence and number of mitotic spindles in lily cells, and interrupt the process of mitosis in root cells of onion (Banerjee and Margulis 1973; Jackson 1969). In the current study, low level of melatonin and naphthalene acetic acid was found

optimum for biomass accumulation. Low levels of melatonin act as growth stimulator in *Lupinus albus* in a similar way like that of auxin (indole acetic acid) that induced the growth of hypocotyls whereas higher concentration inhibited it (Hernandez-Ruiz et al. 2005). In earlier studies on *Avena sativa*, *Triticum aestivum*, *Hordeum vulgare*, *Phalaris canariensis*, the growth promoting activity of melatonin is directly correlated with indole acetic acid concentration (Hernandez-Ruiz et al. 2004). It showed that the addition of auxin and melatonin to culture media might be effective for enhancing biomass and secondary metabolites due to their co-actions.

Effect of melatonin on production of stress enzymes and total protein content, and protease activity

Melatonin is one of important growth regulators and effective antioxidant which directly scavenge toxic free radicals, activate the natural defense enzymes, protect enzymes and protein from oxidative stress, increase the efficiency of mitochondrial transport chain and minimize the generation of toxic free radicals (Tan et al. 2010). In this study, Murashige and Skoog media was incorporated with different concentrations of melatonin alone or in combination with α -naphthalene acetic acid. Both leaf and petiole explants were investigated for production of content of total protein, protease activity, and superoxide dismutase and peroxidase enzymes. Amongst different concentrations of melatonin tested (0.5–2.0 mg/l), highest content of total protein (90.47 μ g BSAE/mg FW) and protease activity

(4.77 U/g FW) were observed in calli obtained from leaf explants on medium augmented with 1.0 mg/l melatonin (Figs. 3 and 4). However, higher levels of superoxide dismutase (3.011 nM/min/mg FW) and peroxidase (1.73 nM/min/mg FW) enzymes were observed on media augmented with lower concentrations (0.5 mg/l) of melatonin (Figs. 5 and 6). It was observed that higher concentrations of melatonin inhibited content of total protein, protease activity, and superoxide dismutase and peroxidase enzymes production. Exogenous melatonin can act as strong antioxidant that directly scavenge reactive oxygen species, reactive nitrogen species and also detoxify diverse chemical contaminants (Arnao and Hernandez-Ruiz 2014). The physiological action of melatonin in various field crops makes them an interesting candidate for antioxidant activity against cold stress, heat, drought, salinity, herbicides, ultra-violet irradiation and chemical pollutants (Arnao and Hernandez-Ruiz 2014; Arnao 2014; Arnao and Hernandez-Ruiz 2009). The antioxidant activity of melatonin has been tested in various field crops. The barley and lupin have shown enhanced vegetative growth against various chemical stressors in the presence of melatonin (Arnao and Hernandez-Ruiz 2009, 2013). Similarly, following melatonin application, the pea plants and red cabbage seed-derived plants have shown excellent growth and development exposed to copper stress (Janas and Posmyk 2013; Tan et al. 2007). Moreover, Lei et al. (2004) reported that melatonin is positively correlated with polyamines

Fig. 3 Effects of melatonin and naphthalene acetic acid supplemented media on content of total protein from leaf and petiole explants in *P. vulgaris*. Data was collected from 3 independent experiments. Mean values with standard errors ($n = 3$) are significantly different at $P < 0.05$

Fig. 4 Effects of melatonin and naphthalene acetic acid supplemented media on protease activity from leaf and petiole explants in *P. vulgaris*. Data was collected from 3 independent experiments. Mean values with standard errors ($n = 3$) are significantly different at $P < 0.05$

Fig. 5 Effects of melatonin and naphthalene acetic acid supplemented media on superoxide dismutase from leaf and petiole explants in *P. vulgaris*. Data was collected from 3 independent experiments. Mean values with standard errors ($n = 3$) are significantly different at $P < 0.05$

Fig. 6 Effects of melatonin and naphthalene acetic acid supplemented media on peroxidase from leaf and petiole explants in *P. vulgaris*. Data was collected from 3 independent experiments. Mean values with standard errors ($n = 3$) are significantly different at $P < 0.05$

synthesis and protect *Daucus carota* cell suspension cultures from cold induced apoptosis.

In comparison, calli obtained from petiole explants showed highest content of total protein (160.8 μg BSAE/mg FW) and protease activity (5.35 U/g FW) on media containing 0.5 mg/l melatonin than control treatments

(96.6 μg BSAE/mg FW and 4.62) (Figs. 3 and 4). Garcia et al. (1997) and Venegas et al. (2012) reported that melatonin protects the fluidity of membrane in animal cells from environmental stresses. The influence of melatonin on cell membrane and protein could be similar in plants but the literature is still limited in this regard (Szafranska et al. 2012; Tan et al. 2012). In contrast to leaf explant, higher superoxide dismutase (3.32 nM/min/mg FW) and peroxidase (1.92 nM/min/mg FW) enzymes production were found in cultures treated with 1.0 mg/l melatonin. Furthermore, the combination of 1.0 and 1.5 mg/l melatonin with 2.0 mg/l naphthalene acetic acid enhanced the content of total protein (270.32 μg BSAE/mg FW) and protease activity (6.44 U/g FW) in cultures obtained from leaf explants (Fig. 3 and 4). While superoxide dismutase (4.11 nM/min/mg-FW) and peroxidase (1.99 nM/min/mg-FW) enzymes were found higher on media containing 0.5 mg/l melatonin and naphthalene acetic acid (Figs. 5 and 6). Similarly, cultures obtained from petiole explants have shown higher content of total protein (168.96) and protease activity (5.6 U/g FW) on media containing combination of 1.0 and 1.5 mg/l melatonin with 2.0 mg/l naphthalene acetic acid as compared to control. The combination of 0.5 mg/l melatonin and 2.0 mg/l naphthalene acetic acid was found suitable for production of superoxide dismutase (2.16 nM/min/mg-FW) and peroxidase (4.77 nM/min/mg-FW) enzymes in cultures obtained from petiole explants.

Melatonin stimulated redox state of cells, reduced the generation of reactive oxygen species and reactive nitrogen species and stabilizing biological membranes in plants like that of animal cells (Catala 2007; Galano et al. 2011; Hardeland 2012; Fischer et al. 2013). Li et al. (2012) observed that pretreated seedling of *Malus hupehensis* with melatonin showed good shoot height, number of leaves and chlorophyll content under the influence of saline stress as compared to untreated plants. They further highlighted that peroxidase, catalase and ascorbate peroxidase activities were induced and sodium and potassium transporters were up-regulated, which would all help to alleviate saline-induced inhibition (Li et al. 2012). Similarly in tomato fruits, the content of melatonin was higher in mature fruits than green ones, which may be related to the protection of fruit against intensive production of reactive oxygen species during ripening (Posmyk et al. 2008). Furthermore, Zhao et al. (2011a, b) documented that before cryopreservation, the pretreatment of calli of *Rhodiola crenulata* with melatonin improved calli survival and protect its tissues from oxidative damage.

It has been concluded from the current experiment that melatonin induced cultures have potential for scale up to a commercial level by different industries to further enhance the production of antioxidative enzymes for multiple

applications. Moreover, the in vitro cultures presented herein can be taken up for further research on other aspects such as mutagenesis, molecular analysis and enhanced production of other secondary metabolites.

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