

Effects of sodium nitroprusside on callus browning of *Ficus religiosa*: an important medicinal plant

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Abstract Tissue browning is a major problem in tissue culturing of woody plants, especially for *Ficus religiosa* which occurs by the accumulation and oxidation of phenolic compounds. This study aimed to determine the effect of different concentrations of sodium nitroprusside on the appearance of callus browning from leaf explants. The results indicate that callus browning was significantly reduced by supplementation of sodium nitroprusside to the MS medium and supplemented with 2.26 μM of 2,4-dichlorophenoxyacetic acid and 0.22 μM of 6-benzyl amino purine. The accumulation of hydrogen peroxide and phenolic compounds in the callus tissues decreased at the 50 μM concentration of sodium nitroprusside. Although catalase and peroxidase activities decreased at the 50 μM concentration, the activity of superoxide dismutase and polyphenol oxidases, as well as proline content, increased exponentially. Sodium nitroprusside could be useful for the formation of non-embryogenic callus with high levels of metabolic activity for the production and isolation of secondary metabolites.

Keywords Nitric oxide · Oxidative enzymes · Proline · Reactive oxygen species

Introduction

Ficus religiosa L. provides a traditional remedy for various diseases because of its broad range of secondary metabolites such as lupin 3-one, bergenin, lanosterol, methyl oleanolate, stigmasterol, amides, caffeic acid, flavonoids, and β -sitosterol (Singh et al. 2011; Salmi and Hesami 2016). One of the most useful techniques for the production of secondary metabolites of *Ficus religiosa* is tissue culturing (Hesami and Daneshvar 2018a; Hesami et al. 2018c). The primary step during the production of secondary metabolites is callus culture that is divided into two specific types: embryogenic or non-embryogenic (Hesami et al. 2017b, 2018b; Niazian et al. 2017). The most appropriate is a non-embryogenic callus culture consisting of the homogeneous bulk of dedifferentiated cells (Jedinák et al. 2004). Extreme callus browning, an interfering phenomenon in in vitro cultures, is usually observed during culturing of *F. religiosa* (Hesami and Daneshvar 2018b). Callus browning is one of the most significant problems in the production and isolation of secondary metabolites for this valuable medicinal plant.

Callus browning can inhibit plant growth and reduce cell proliferation (Ling et al. 2007; Chugh et al. 2009; Mondal et al. 2014). Yoruk and Marshall (2003) indicated that there are many elements responsible for callus browning in the phenolic oxidation process such as phenolic compounds, the action and presence of reactive oxygen species (ROS), and oxidative enzymes. One of the common alarming signals for changing the expression of genes and metabolism against stress is the accumulation of hydrogen

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peroxide which may limit plant growth and lead to cell death (Foyer et al. 1994). Generally, ROS can be produced during the normal processes of plant metabolism such as respiration and photosynthesis. The ROS level can also be increased exponentially under various abiotic stresses such as drought, salinity, UV light, and cold (Sharma et al. 2012; Tripathy and Oelmüller 2012). Furthermore, some mechanical damages during culturing, especially in the explant preparation step such as wounding, are important factors for increased ROS that lead to callus browning (Wang et al. 2011). There are various cellular structures and organelles responsible for producing and scavenging ROS under stress, such as plasmalemma, chloroplasts, the endoplasmic reticulum (ER), mitochondria, and cell walls (Gill and Tuteja 2010). Oxidative damage in many cellular components, for example, plasmalemma disintegration, breakage of the nuclear envelope, and chloroplast deformation, results in the accumulation of ROS that leads to peroxidation of lipids, hydroxylation of nucleic acids, and oxidation of proteins (Mittler 2002; Gill and Tuteja 2010). Excessive accumulation of ROS accompanied with the disorganization of organelles, results in limiting plant growth and finally cell death (Kratsch and Wise 2000; Wi et al. 2005; Wahid et al. 2007; Sharma et al. 2012) that can be demonstrated by the browning of callus.

In the study by Liu and Chen (2010), it became clear that during callus formation, the production of various types of ROS such as superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$) increased significantly. Oxidative stress, which is shown by excess production of ROS, has a negative influence on plant growth and development (de Pinto and De Gara 2004; Pitzschke et al. 2009). However, there are many types of oxidative enzymes involved in enzymatic browning such as polyphenol oxidase (PPO), peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD) (Xu et al. 2005; Yingsanga et al. 2008).

Sodium nitroprusside (SNP), by releasing nitric oxide (NO), a ubiquitous bioactive molecule, is a highly reactive gas that serves as an important molecule in signal transduction in plants under stress (Arasimowicz and Floryszak-Wieczorek 2007). The effect of nitric oxide on various types of cells reveals that this molecule plays a significant role as an antioxidant (Qiao and Fan 2008). Sodium nitroprusside, pH-dependent, releases nitric oxide which reduces senescence and induces plant growth and development (Kolbert et al. 2008). This valuable molecule is a new member of plant growth regulators (Jimenez-Quesada et al. 2017) that might scavenge reactive oxygen species (Laspina et al. 2005). Furthermore, the synthesis of nitric oxide was promoted by cytokinins in cell culturing of parsley, rockcress, and tobacco (Tun et al. 2001). Recently, nitric oxide has been used for developing plant tissue

culture protocols (Rico-Lemus and Rodríguez-Garay 2014). Kalra and Babbar (2010) reported that nitric oxide could enhance the regeneration response by increasing the number of meristems, and suggested that it could regulate gene expression related to the differentiation of meristems. Sarropoulou and Maloupa (2017) found that nitric oxide could exert a powerful impact on cell division and could be involved in shoot organogenesis and proliferation. Han et al. (2009) and Sarropoulou et al. (2014) showed that *in vitro* shoot proliferation as well as root formation of plantlets were significantly promoted by applying SNP to the Murashige and Skoog (MS) medium for Chinese crabapple and cherry rootstocks. Xu et al. (2009) reported that nitric oxide exerted a strong impact on callus induction and browning of Chinese yam.

Nitric oxide promotes plant tolerance against stress by reducing the transportation of Na^+ from root to shoots and the neutralization of the negative effects of oxidative damage in membranes (Guo et al. 2009; Corpas and Barroso 2017). Based on Sarropoulou et al. (2016), the positive effects of sodium nitroprusside on growth of cherry was determined under *in vitro* conditions. The resistance of *Pinus eldarica* Ten. to oxidative damage and the improvement of its growth traits increased by using sodium nitroprusside and salicylic acid (Zamani et al. 2014). In another study, Uchida et al. (2002) demonstrated that H_2O_2 and nitric oxide serve as significant signaling molecules for enhancing tolerance against abiotic stress in rice. Nitric oxide can improve tolerance against oxidative stress in *Lycopersicon esculentum* Mill. by increasing the activity of antioxidant enzymes (Hayat et al. 2012). Nitric oxide might serve as an antioxidant for reducing toxicity promoted by H_2O_2 (Laspina et al. 2005). As a result, this led us to study the effect of sodium nitroprusside (SNP) in controlling the browning of callus.

The aim of this study was to establish and develop appropriate and optimal protocols for the callus induction of *F. religiosa*. No previous study reports on the effect of sodium nitroprusside on callus browning of *F. religiosa*. This study focused on the effect of different concentrations of sodium nitroprusside on callus browning from the leaf explants of *F. religiosa*.

Materials and methods

Plant materials and sterilization

The fruits were collected from 45 to 50-year-old *F. religiosa* trees on the campus of the Ramin Agriculture and Natural Resources University, Khuzestan, Iran, washed with tap water for 30 min and washed again after cleaning with a liquid soap solution. Further surface sterilization

was applied in a laminar air flow cabinet. The seeds were surface sterilized with 70% aqueous ethanol for 10 s, dipped in 10% (v/v) sodium hypochlorite (NaClO) solution for 5 min, and then washed three times in sterilized distilled water. The seeds were inoculated on one-tenth strength MS medium, germinated for 4 weeks, and the leaf explants used as a source of explants.

Media and culture condition

To determine the effect of sodium nitroprusside on callus browning, 5-mm leaf segments (abaxial side) from 4-week-old *in vitro* grown seedlings (Fig. 1a) were inoculated on MS basal medium containing 0.6% (w/v) agar (Duchefa Biochemie, Netherlands), 3% (w/v) sucrose, as well as 2.26 μM 2,4-D and 0.22 μM BAP. The pH was adjusted to 5.8 ± 0.2 . The medium was autoclaved at 121 $^{\circ}\text{C}$ for 15 min at 15 psi. Subsequently, sodium nitroprusside, at different levels (10.0, 20.0, 30.0, 40.0, and 50.0 μM), was added to the basal medium via 0.2 μm filter, and distributed into 200 ml jars containing 15 ml of culture medium. All cultures were kept under 16-h light and 8-h dark photoperiod at ($25 \pm 2^{\circ}\text{C}$) with a light intensity of 45 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from cool white fluorescent lights. Calluses were subcultured every 3 weeks on the same composition of fresh MS medium.

Determination of H_2O_2 levels, activities of antioxidative enzymes, accumulations of proline, and total phenolic contents

Eight weeks after culturing, H_2O_2 levels, accumulations of proline in callus tissues, and the phenolic contents were evaluated based on Velikova et al. (2000), Errabii et al. (2007), and Park et al. (2006), respectively. Activities of antioxidative enzymes such as SOD (Beauchamp and Fridovich 1971), CAT (Cakmak and Horst 1991), POD (Nickel and Cunningham 1969), and PPO (Ramamoorthy et al. 2002) were measured accordingly.

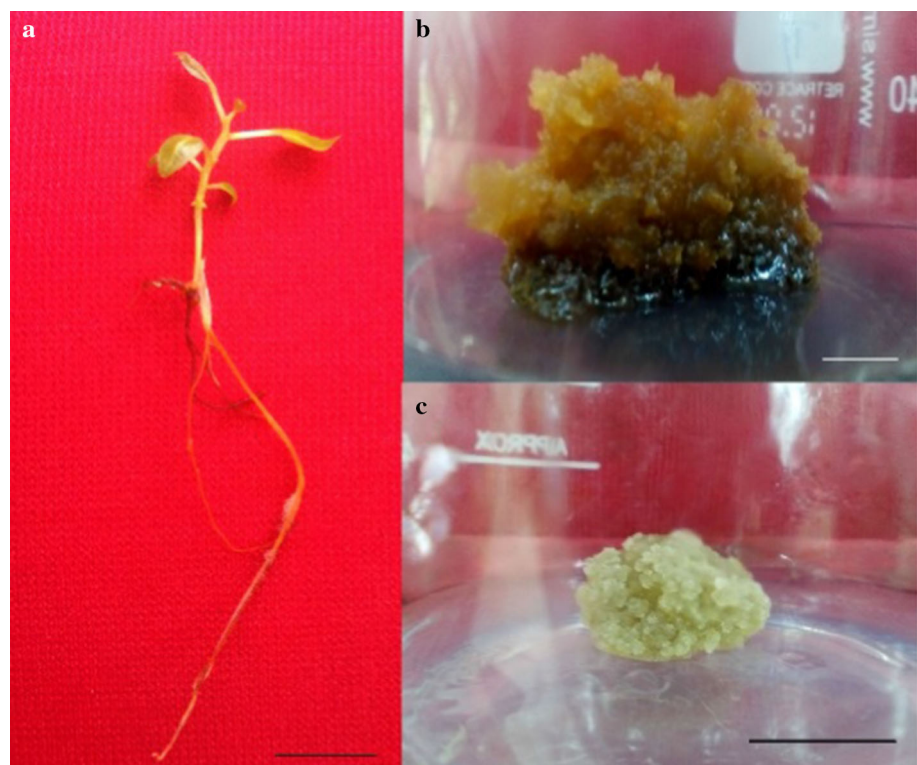
Statistical analyses

The experiments were carried out in a completely randomized design of 10 replicates per treatment and each treatment repeated in three sets. The data were analyzed by Analysis of Variance (ANOVA) followed by Duncan's multiple range test (Duncan 1955) at $P < 0.05$. Data analysis used SAS version 9.3.

Results and discussion

One of the most significant problems during the *in vitro* culturing of *F. religiosa* is callus browning which is associated with the overproduction of phenolic compounds and H_2O_2 in the tissues.

Fig. 1 Callus formation from seedling-derived leaf segments of *Ficus religiosa*; **a** 4-week-old seedling from *in vitro* seed germination; **b** brown callus on MS medium supplemented with 2.26 μM 2,4-D and 0.22 μM BAP after 8 weeks; **c** yellow and friable callus on MS medium containing 2.26 μM 2,4-D and 0.22 μM BAP along with 50 μM SNP after 8 weeks; Bar = 0.5 cm



Since various phenolic compounds exist in *F. religiosa* which have a pharmaceutical value (Hesami et al. 2017a), the callus culture can be useful for the production and isolation of secondary metabolites (Singh et al. 2011). Thus the callus browning caused by the oxidation of phenolic compounds not only leads to plant death but also significantly reduces the production of phenolic compounds by limiting its biosynthetic capacity (Ko et al. 2009). One of the most important elements are the phenolic compounds because they serve as substrates for oxidative enzymes. Based on our results, the total phenolic content in the control callus was significantly higher than in the H₂O₂ treatments (Figs. 1, 2). By increasing the concentration of sodium nitroprusside, the level of browning decreased significantly; 50 μ M sodium nitroprusside was the most effective concentration (Fig. 2). Furthermore, the yellowish friable callus was also obtained at this concentration. However, the over-production of phenolic compounds negatively influenced callus growth and high amounts of phenolic compounds resulted in tissue browning. This has also been reported for *Cicer arietinum* L. (Naz et al. 2008) as well as for *Pistacia vera* L. (Leng et al. 2009). According to a study by Xu et al. (2009), sodium nitroprusside was used completely for reducing or inhibiting oxidative browning in callus cultures of *Dioscorea opposita* Thunb. Nitric oxide also can scavenge the production of oxygen radicals where the tissue is wounded, and one of the important roles of nitric oxide in plants is to act as a protector against oxygen radicals (Hesami et al. 2018a).

Polyphenol oxidase (PPO) is an important defense enzyme against oxidative stress in various plant species and is located in the thylakoid membrane of the chloroplasts (Mayer 2006). The major role of PPO is in the enzymatic oxidation of phenolic compounds which result in necrosis and death of explants (Mayer 2006). Where PPO acts as a catalyzer in the oxidation of phenolic

compounds to form quinone compounds, cell browning occurs (Dehon et al. 2002). Afterwards, quinones can polymerize to produce brown pigments called melanins. Phenols can be converted into quinones in the presence of metal ions. Nitric oxide can convert compounds by chelating metal ions to react with phenols to prevent the changing of phenols to quinones (Flora 2009). According to our study, by increasing the concentration of sodium nitroprusside, PPO activity decreased significantly in comparison with the control treatment (Fig. 3). In the medium with 50 μ M sodium nitroprusside, the maximum decreased activity occurred (Fig. 3). Thus, it can be interpreted that nitric oxide has a special advantage for improving plant growth in in vitro conditions as well as for managing problems occurring by phenolic compounds. Nitric oxide can be helpful for maintaining the biosynthetic potential of the callus and for leading to the efficient production of phenolic compounds useful in medicine.

Based on our results, changes in the concentration of sodium nitroprusside can affect the browning of callus. Five days after culturing plants in the callus medium, the surfaces of explants turned brown. At the 50 μ M sodium nitroprusside level, callus browning was suppressed significantly. It appears that browning is closely associated with the production of H₂O₂ in plant cells. The next step of this study was to evaluate the effects of sodium nitroprusside on the over accumulation of H₂O₂ in explants. Our results indicate that over accumulation was reduced significantly by increasing the levels of sodium nitroprusside (Fig. 4). The accumulation of H₂O₂ was highest in the medium without sodium nitroprusside, but significantly decreased with increase in the level of sodium nitroprusside from 10 to 50 μ M (Fig. 4). Foyer et al. (1994) suggested the production of H₂O₂ might act as a common alarm signal leading to modification of gene expression and metabolism. Consequently, if nitric oxide reacts with H₂O₂, this could potentially abrogate hydrogen peroxide

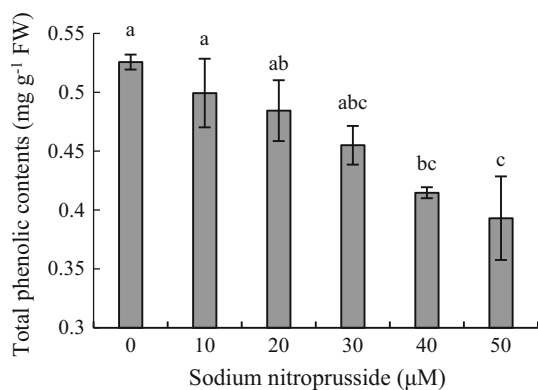


Fig. 2 Effect of sodium nitroprusside (SNP) on total phenolic contents of *Ficus religiosa* callus (means followed by the same letter are not significantly different at $P < 0.05$ as determined by Duncan's multiple range test)

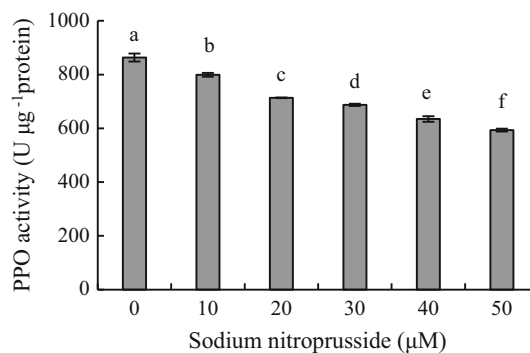


Fig. 3 Effect of sodium nitroprusside (SNP) on polyphenol oxidase (PPO) activity of *Ficus religiosa* callus (means followed by the same letter were not significantly different at $P < 0.05$ as determined by Duncan's multiple range test)

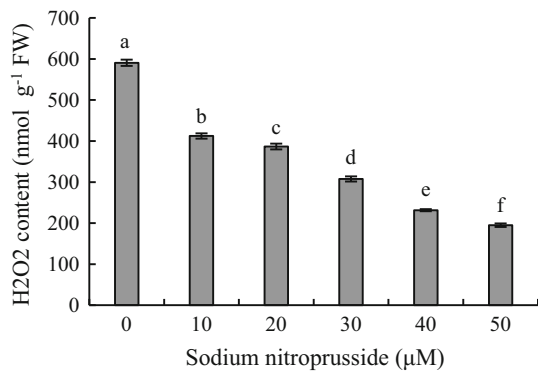


Fig. 4 Effect of sodium nitroprusside (SNP) on hydrogen peroxide (H₂O₂) content of *Ficus religiosa* callus (means followed by the same letter were not significantly different at $P < 0.05$ as determined by Duncan's multiple range test)

signaling (Neill et al. 2003). On the other hand, the overproduction of H₂O₂ results in inhibiting plant growth and in cell death. Nitric oxide might serve as an antioxidant for reducing toxicity promoted by H₂O₂ (Laspina et al. 2005). A correlation between H₂O₂, NO, and antioxidant levels has also been demonstrated by de Pinto et al. (2002). In tobacco BY-2 cells, neither NO nor H₂O₂ alone at low concentrations had any effect on programmed cell death (PCD) or on the activity of phenylalanine ammonia-lyase (PAL). However, treatment with both H₂O₂ and NO together induced a substantial increase in cell death with characteristics of PCD, as well as PAL activity. Moreover, this treatment also caused an increase in the activities of enzymes reducing ascorbate and glutathione (de Pinto et al. 2002), implying that both H₂O₂ and NO regulate cellular antioxidant levels to effect PCD, at least in some systems (Neill et al. 2003).

The antioxidative systems in plants are divided into two distinct parts; non-enzymatic antioxidants such as proline which is detoxified ROS, and enzyme antioxidants such as catalase (CAT) and superoxide dismutase (SOD) which convert ROS into water, oxygen, and hydrogen peroxide. This antioxidant system can scavenge various types of ROS and protect cells and tissues against oxidative stress (Corpas and Barroso 2017). In the present study, SOD activity increased in the 50 μM SNP concentration (Fig. 5) but CAT (Fig. 6) and POD (Fig. 7) activities decreased in this treatment. Proline is one of the major non-enzymatic antioxidants in plants. In the present study, proline content increased from 10 to 50 μM concentrations of sodium nitroprusside, i.e., the highest level was at 50 μM SNP (Fig. 8). Superoxide dismutase converts O₂^{·-} to H₂O₂ and O₂ in the chloroplasts, mitochondria, and cytosol (Xu et al. 2009). The high activity of superoxide dismutase may protect callus tissues against oxidative stress. Xu et al. (2009) indicated that 40 μM sodium nitroprusside

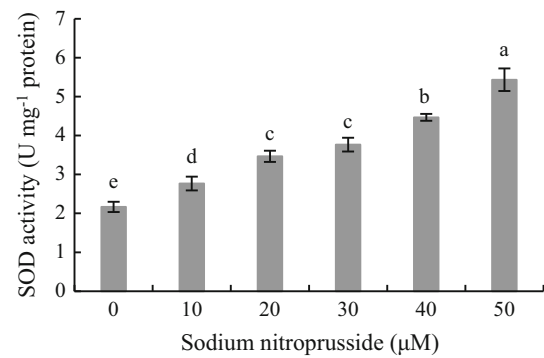


Fig. 5 Effect of sodium nitroprusside (SNP) on superoxide dismutase (SOD) activity of *Ficus religiosa* callus (means followed by the same letter were not significantly different at $P < 0.05$ as determined by Duncan's multiple range test)

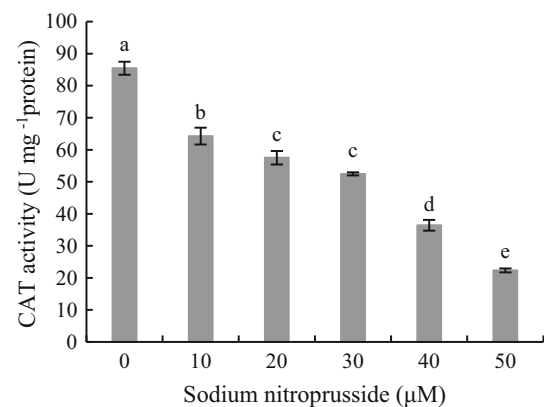


Fig. 6 Effect of sodium nitroprusside (SNP) on catalase (CAT) activity of *Ficus religiosa* callus (means followed by the same letter were not significantly different at $P < 0.05$ as determined by Duncan's multiple range test)

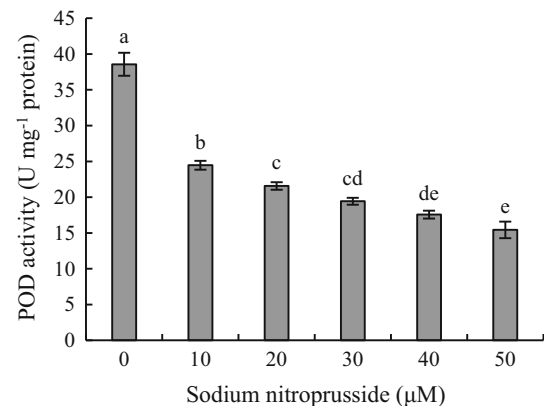


Fig. 7 Effect of sodium nitroprusside (SNP) on peroxidase (POD) activity of *Ficus religiosa* callus (means followed by the same letter were not significantly different at $P < 0.05$ as determined by Duncan's multiple range test)

significantly increased the activity of superoxide dismutase in *Dioscorea opposita*. Catalase (CAT) is a key enzyme

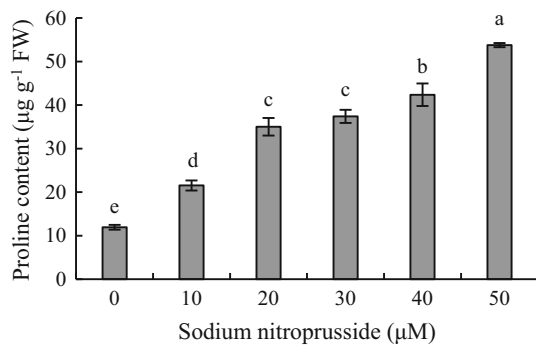


Fig. 8 Effect of sodium nitroprusside (SNP) on proline contents of *Ficus religiosa* callus (means followed by the same letter were not significantly different at $P < 0.05$ as determined by Duncan's multiple range test)

that converts H_2O_2 to H_2O and O_2 , and peroxidase (POD) is an important component of the enzymatic antioxidant system. The activities of CAT and POD decreased at the 10–50 μM concentration of sodium nitroprusside. Our results are in line with those of Corpas and Barroso (2017) who indicated that nitric oxide might inhibit CAT and POD activities. Parani et al. (2004) reported that the gene expression of POD (*At2g37130*) down-regulated at 100 μM of sodium nitroprusside in *Arabidopsis*. In general, sodium nitroprusside decreased CAT and POD activities, while proline levels increased significantly. Proline, as the major non-enzymatic defense system, could be responsible for detoxifying ROS (reactive oxygen species) produced under biotic and abiotic stresses. Furthermore, the accumulation of proline could change the accumulation of various transcripts related to gene expression and cell division control (Maggio et al. 2002). Thus, proline accumulation along with sodium nitroprusside (SNP) induces cell proliferation and enhances the antioxidative activities of plants in tissue cultures.

Conclusion

Ficus religiosa is an important medicinal plant owing to its wide range of secondary metabolites. Therefore, callus culturing of this species would be useful for mass production of secondary metabolites. However, tissue browning is a major problem during culturing of this plant as it is with other woody plants. Our results indicate that sodium nitroprusside (SNP) has broad supplementation in culturing of *F. religiosa* where browning limits callus induction. SNP also could be helpful for the formation of non-embryogenic callus with high levels of metabolic activity for the production and isolation of secondary metabolites.

Authors' contribution All authors listed have made substantial, direct and intellectual contribution to the work, and have approved it for publication.

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