



Reactive oxygen species, nitric oxide and plant cell death associated with caspase-like protease activity during somatic embryogenesis in *Fraxinus mandshurica*

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Abstract Programmed cell death occurs in browning explants of *Fraxinus mandshurica* during somatic embryogenesis, but the underlying mechanism is unclear. In this study, single cotyledons of zygotic embryos of *F. mandshurica* were used as explants. Mitochondrial structure and function, caspase-3-like protease activity, hydrogen peroxide metabolism, and nitric oxide accumulation induced by high concentrations of sucrose and plant growth regulators were studied. The results show that plant growth regulators induced somatic embryogenesis and also promoted explant browning. High sucrose concentrations had similar effects. High concentrations of sucrose and plant growth regulators

led to the accumulation of hydrogen peroxide and nitric oxide which induced changes in mitochondrial structure and function such as modifications in mitochondrial morphology, increased membrane permeability, decreased membrane potential, and the release of cytochrome c into the cytoplasm. An increase in caspase-3-like protease activity triggered programmed cell death in some browning explant cells. During somatic embryogenesis there were increased activities of superoxide dismutase, peroxidase, and catalase, which are associated with hydrogen peroxide metabolism and jointly maintain reactive oxygen species levels. Intracellular nitric oxide synthase and nitrate reductase activities were not significantly correlated with nitric oxide content. Instead, intracellular nitric oxide may be derived from non-enzymatic reactions. Our results indicate that hydrogen peroxide and nitric oxide may function as signals, playing key roles in somatic embryogenesis and programmed cell death of explant cells of *F. mandshurica*. The interaction between nitric oxide and reactive oxygen species determines the occurrence of programmed cell death in explant cells; somatic embryogenesis and programmed cell death are positively regulated by hydrogen peroxide. However, the regulation of nitric oxide is complex.

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Introduction

Plant somatic embryogenesis (SE) is good evidence that plant cells are totipotent, capable of giving rise to any cell type. A key step in SE is the transformation of cells from somatic cells to embryonic cells (Guan et al. 2016). In some plants, programmed cell death (PCD) has been observed during the formation of embryogenic cells (Petruzza et al. 2009; Rodríguez-Serrano et al. 2012; Huang et al. 2014a). However, the relationship between PCD and embryogenic cell formation, as well as its regulation mechanism, are unclear. Mitochondria regulate and amplify apoptotic signals and play a role in plant PCD processes (Zhao et al. 2018). The morphological structure of mitochondria changes when PCD occurs; mitochondria blur, their internal structure swells, and finally they rupture (Wang et al. 2009; Doorn et al. 2011). At the same time, caspase-3 activity, which is closely associated with PCD, increases (Petruzza et al. 2009). Some studies have shown that hydrogen peroxide (H_2O_2) and nitric oxide (NO) are involved in PCD during plant embryogenic cell formation (Huang et al. 2014a; Zhou et al. 2016). Although a relationship among PCD, NO, and H_2O_2 has been observed, their roles and relationships in plant embryogenic cell formation are not well understood.

Although SE has been achieved in several woody species, this form of propagation remains difficult for some mature woody plants. *Fraxinus mandshurica* Rupr., an important member of the genus *Fraxinus*, is distributed in temperate forest ecosystems in northeastern China, and has a high timber value (Gu et al. 2015). Technology for SE in this species has been studied for 15 years, and a direct SE system using zygotic cotyledons as explants has been established. Seedlings regenerated using this technology have been planted in the field and continue to grow well (Yang et al. 2013). In the process of embryogenic cell production in *F. mandshurica*, however, some explants show browning, which can lead to the death of the cells and limit the production of regenerated plants. In addition, the browning of explants seems likely to be related to programmed cell death because the levels of intracellular reactive oxygen species (ROS) increase during the browning process (Liu et al. 2015; Yang et al. 2019). There is a need to explore the roles of signalling molecules such as ROS in the transformation of somatic cells into embryogenic cells, and to clarify their relationships with PCD.

In this study, changes were monitored in mitochondrial structure and function, caspase-3 enzyme activity, intracellular H_2O_2 metabolism, and NO accumulation during SE in explants under high concentrations of sucrose and in response to plant growth regulators (PGRs). The objectives were to explore: (1) the functions of high concentrations of sucrose in SE and PCD of explant cells; and, (2) the functions of, and relationships between, H_2O_2 and NO in SE and

PCD of explant cells. Our results should provide insights into the roles of signalling molecules (e.g., ROS) in the process of the transformation of somatic cells into embryogenic cells and their relationship with PCD, and may provide experimental evidence for the regulation of SE and PCD in *F. mandshurica*.

Materials and methods

Experimental materials

Mature dehydrated seeds (Fig. 1a) were collected in late September 2015 from a mature mother tree of *F. mandshurica* growing at the Shanhetun Forestry Bureau, Wuchang City, Heilongjiang Province. Single cotyledons of zygotic embryos were used as explants.

Methods

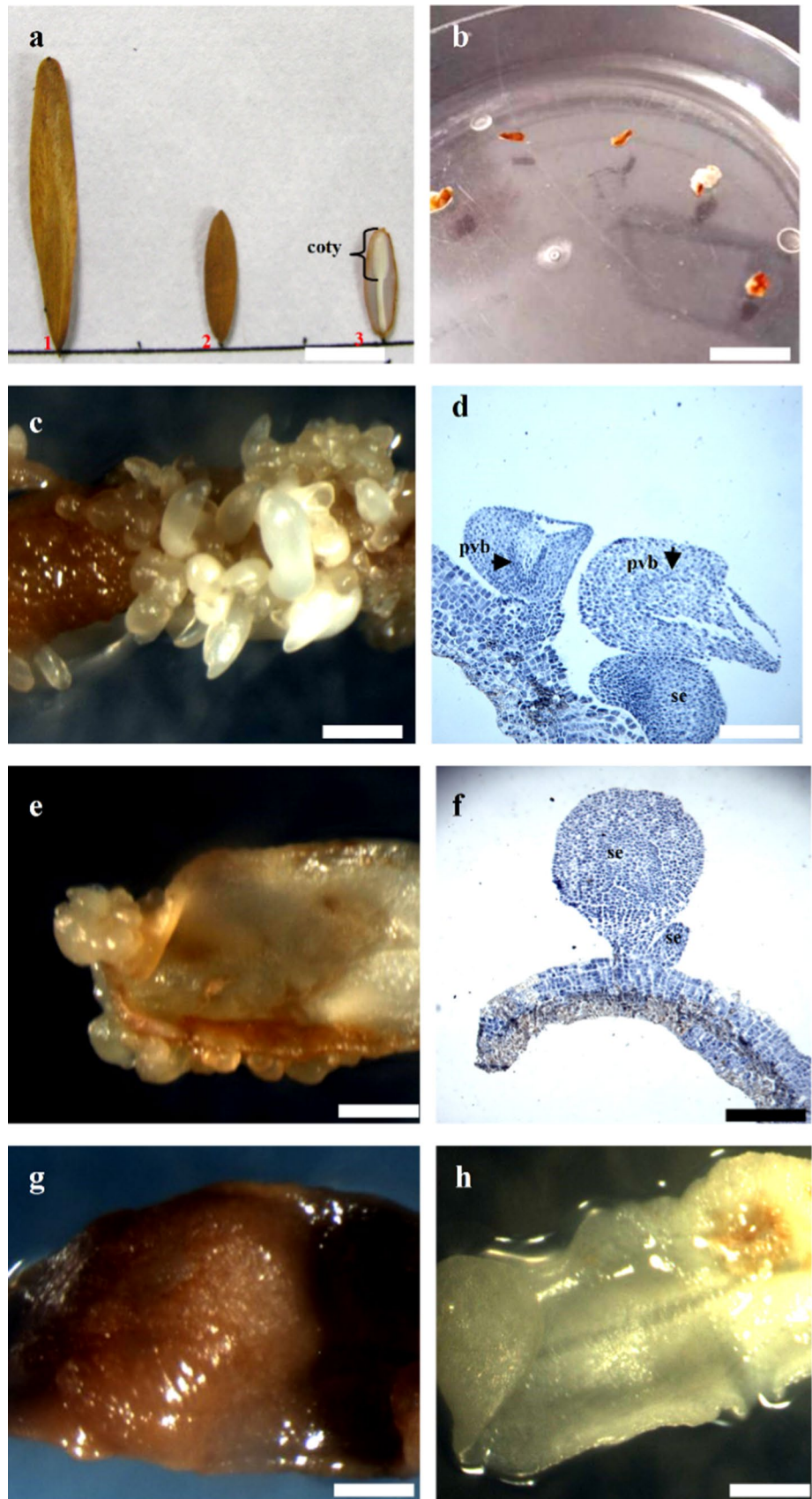
Effect of high sucrose and PGRs concentrations on somatic embryogenesis

Material pretreatment Based on Yang et al. (2013), the collected seeds were mixed evenly and their pericarps removed. The seeds were soaked in running tap water for two days, then agitated in 70% (v/v) aqueous ethanol for 1 min, surface sterilized in 3% (v/v) aqueous sodium hypochlorite for 15 min, and finally rinsed five times with sterile distilled water. Single cotyledons from zygotic embryos were inoculated onto the surface of the medium, with the inner side of the cotyledon in contact with the medium.

Somatic embryo induction and extraction methods The methods for SE induction and extraction were those described by Yang et al. (2013), with some modifications. The basic medium for SE induction was 1/2-strength Murashige and Skoog medium (1/2-MS), with the addition of 26.84 μ M naphthalene acetic acid (NAA) and 8.88 μ M benzyl adenine (BA) or without PGRs, 400 mg·L⁻¹ casein hydrolysate (CH), 75 or 20 g·L⁻¹ sucrose, and 6.5 g·L⁻¹ agar (Beijing Aoboxing Biotechnology Co., Ltd., Beijing, China). The pH of all media was adjusted to 5.8 before autoclaving. The explants were inoculated onto the different media and cultured for 60 d in the dark at 23–25 °C. Each treatment consisted of 10 petri dishes, each inoculated with 10 explants, and each treatment repeated three times. On day 60, the occurrence of somatic embryos was recorded, and samples were collected for paraffin sectioning (Yang et al. 2019).

Cultured materials collected at the beginning and on day 13 were observed by transmission electron microscopy. Mitochondrial function indexes, caspase-like enzyme and

Fig. 1 Explant browning and somatic embryogenesis of *Fraxinus mandshurica* at day 60 of culture. **a** mature samara (1), endosperm-coated embryos (2) and cotyledon-type embryos filled with embryonic cavities (3); **b–d** single cotyledons cultured on medium with $75 \text{ g}\cdot\text{L}^{-1}$ sucrose + $26.84 \mu\text{M}$ NAA + $8.88 \mu\text{M}$ BA. **b** explant browning. **c** somatic embryos in different developmental stages. **d** “Y” shaped vascular tissue of somatic embryo; **e** and **f** single cotyledon cultured on medium with $20 \text{ g}\cdot\text{L}^{-1}$ sucrose + $26.84 \mu\text{M}$ NAA + $8.88 \mu\text{M}$ BA. **e** spherical embryos; **f** spherical somatic embryo with radiation symmetry structure. **g** single cotyledon cultured on medium with $75 \text{ g}\cdot\text{L}^{-1}$ sucrose. The explant turned brown, but there were no somatic embryos. **h** single cotyledon cultured on medium with $20 \text{ g}\cdot\text{L}^{-1}$ sucrose. The explant did not turn brown, and no somatic embryos were observed. coty: cotyledons; se: somatic embryo; pvb: primary vascular bundle; bar = 1 cm (**a** and **b**), 5 mm (**c**, **e**, **g**, **h**), 1.25 mm (**d**), 1 mm (**f**)



antioxidant enzyme activities, intracellular H_2O_2 and NO contents, and the activities of NO synthesis-related enzymes were determined on days 5, 7, 9, 11 and 13. Samples were randomly selected for the measurements of each parameter.

Effects of high sucrose and PGRs concentrations on PCD

Sample preparation for transmission electron microscope observations Cultured materials were collected at different times, cut into 3 mm long \times 1 mm wide pieces, immediately placed in 2.5% (v/v) glutaraldehyde solution and kept at 0–4 °C in the dark for 12–48 h. The samples were then removed and washed three times with 0.1 mmol·L⁻¹ phosphate buffer (pH 6.8) for 15 min each time, followed by the addition of 1% (v/v) osmic acid solution and further incubation for 4 h. Samples were finally rinsed three times with 0.1 mmol·L⁻¹ phosphate buffer (pH 6.8) for 15 min each time. The fixed samples were dehydrated in successive steps by immersion in different concentrations of ethanol: 50% (v/v) \rightarrow 70% (v/v) \rightarrow 90% (v/v) \rightarrow 100% (v/v) ethanol, for 20 min in each followed by immersion in a 1 : 1 (v/v) mixture of 100% ethanol and 100% acetone for 20 min, and finally in 100% acetone for 10 min. The dehydration temperature was 0–4 °C. For immersion and embedding, pure acetone was mixed with an embedding agent (domestic epoxy resin 618) = 1 : 1 (v/v), 1 : 2 (v/v) and 1 : 3 (v/v) as a penetrant for soaking. For each ratio, the permeation treatment time was 1, 5, and 24 h, respectively, at room temperature. Samples were immediately placed in a 1.5 mL centrifuge tube, and penetrant was added. The embedding agent was injected into the hole of the embedding block and the sample removed from the centrifuge tube and placed in the hole of the embedding block. The sample was polymerized in an incubator at 60 °C for 48 h, removed, and the block and light mirror positioning were adjusted. Sectioning was carried out with a LKB-5 microtome, to an approximate thickness of 50–70 nm. The sample was subsequently double-dyed with lead citrate and 2% uranyl acetate, and observed and photographed under a Tianmei Hitachi H-7650 electron microscope (Hitachi Ltd., Tokyo, Japan).

Analysis of mitochondrial function indicators Mitochondria were extracted according to Huang et al. (2014b). In a pre-cooled mortar, 0.3 g samples were ground with 2 mL extraction buffer [0.05 mol L⁻¹ tris-HCl buffer (pH 7.4), 1 mmol L⁻¹ ethylenediaminetetraacetic acid disodium salt (EDTA-Na), 0.4 mol L⁻¹ sucrose] into a homogenate. This was centrifuged at 1,800 g for 15 min at 4 °C. The supernatant was collected and then centrifuged at 5,400 g for 15 min at 4 °C. The precipitate was washed three times with extraction buffer and suspended in the same buffer but without EDTA-Na. The mitochondrial concentration was expressed as mitochondrial protein content (mg), and protein content

was determined by the Coomassie brilliant blue method. Mitochondrial membrane permeability analysis was carried out according to Huang et al. (2014b), and mitochondrial membrane potential and Cytochrome c/a were determined following Braidot et al. (1998) and Tonshin et al. (2003), respectively.

Intracellular caspase-3-like protease assay The assay was carried out according to the KGI caspase-3-like protease activity spectrophotometric detection kit (KGA203F, Jiangsu Kaiji Biotechnology Co., Ltd., Jiangsu, China). The fresh material was washed twice with phosphate-buffered saline and placed in a mortar in an ice bath. Subsequently, 150–200 μ L pre-cooled lysis buffer with 0.5 μ L DTT per 50 μ L lysis buffer added before use, and the mixture was vortexed twice for 5 min each time. The supernatant, containing the lysed protein, was transferred into a new pre-cooled centrifuge tube and placed on ice. A small amount of supernatant (1–2 μ L) was collected and the protein concentration was determined according to Bradford (1976). For each sample, 50 μ L supernatant containing 100–200 μ g protein was collected. If the volume was less than 50 μ L, 10 mmol·L⁻¹ PBS (pH 7.2–7.6) was added to obtain a total volume of 50 μ L (all groups were measured and compared based on the same amount of protein). Subsequently, 50 μ L 2 \times reaction buffer, with 0.5 μ L DTT added per 50 μ L 2 \times reaction buffer before use, and 5 μ L caspase-3-like protease active substrate were added. The mixture was incubated for 4 h at 37 °C in the dark. Absorbance was measured with a spectrophotometer (Ultrospec 2100 pro, Amersham Pharmacia Biotech, Little Chalfont, UK) at λ =405 nm. Caspase-3-like protease activity was determined as the multiple in the optical density (OD) of apoptotic cells over that of the negative control. The blank control contained a mixture of 50 μ L lysis buffer and 50 μ L reaction buffer.

Effect of high concentration of sucrose and PGRs on H_2O_2 metabolism

Intracellular H_2O_2 levels were determined according to Zhan et al. (2014). The 0.5 g samples were homogenized in an ice bath with 2 mL 0.1% (w/v) cold trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 g for 15 min at 4 °C and 0.5 mL of the supernatant was added to 0.5 mL of 10 mmol·L⁻¹ potassium phosphate buffer (pH 7.0) and freshly prepared 1 mL of 1 mol·L⁻¹ potassium iodide (KI) was added to 10 mmol L⁻¹ potassium phosphate buffer (pH 7.0). The reaction mixture was then analyzed for absorbance at λ =390 nm, and H_2O_2 concentration was obtained using standard curve. The results are expressed as μ mol·g⁻¹ Pro.

For the assessment of antioxidant enzymes, 0.25 g fresh samples were crushed with 50 mmol·L⁻¹ phosphate buffer (pH 7.8). The homogenate was centrifuged at 12,000 g

for 20 min at 4 °C to produce the supernatant. Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities were based on Zhan et al. (2014).

SOD activity was determined by nitro blue tetrazolium chloride (NBT) photoreduction. A 50 µL supernatant was added to the reaction solution containing 1.5 mL of 50 mmol L⁻¹ potassium phosphate buffer (pH 7.8), 0.3 mL 130 mmol L⁻¹ methionine, 0.3 mL 750 µmol L⁻¹ NBT, 0.3 mL 100 µmol L⁻¹ EDTA-Na₂, 0.3 mL 20 µmol·L⁻¹ riboflavin, and 0.3 mL sterile deionized water. The mixture was positioned 30 cm away from an iodine lamp with 800 µmol·m⁻²·s⁻¹ for 15 min at 25 °C. The reaction mixture was then analyzed for absorbance at 560 nm. One unit of SOD is defined by the amount of enzyme that inhibits NBT photo-reduction by 50%. SOD activity was expressed as unit·g⁻¹ Pro.

POD activity was detected by the guaiacol oxidation method. A 50 µL supernatant was added to a reaction solution containing 1.0 mL 30% H₂O₂ (v/v), 1.0 mL of 0.2 mol L⁻¹ potassium phosphate buffer (pH 6.0), and 0.95 mL 0.2% guaiacol. The mixture was then analyzed for absorbance at 470 nm. One unit of POD was defined as an OD of 0.01. The specific activity of POD was given as unit·g⁻¹ Pro·min⁻¹.

CAT activity was assayed by the decomposition of H₂O₂. A 100 µL supernatant was mixed with a reaction solution containing 1.9 mL of 0.1 mol·L⁻¹ potassium phosphate buffer (pH 7.0) and 1 mL 0.2% H₂O₂ (v/v). The decrease in H₂O₂ was monitored at 240 nm and quantified by its molar extinction coefficient. CAT activity was expressed as mg H₂O₂·g⁻¹ Pro·min⁻¹.

Effect of high concentration of sucrose and PGRs on NO synthesis

Nitric oxide content was determined according to Libourel et al. (2006). Half gram samples were homogenized in an ice bath with 2 mL cold sterile deionized water (pH 8.0). A 50.0 µL of 0.5 mol·L⁻¹ sodium hydroxide (NaOH) and 10.0 µL of 0.4 mol·L⁻¹ zinc sulfate (ZnSO₄) were added to the homogenate. The mixture was placed in 60 °C water for 10 min.

The mixture was then centrifuged at 5,400 g for 15 min at 4 °C and 1.0 mL of the supernatant was added to a 1.0 mL reaction solution containing 1% sulfanilamide (w/v, in 5% phosphoric acid) and 0.1% N-1-naphthyl-aminophylline flurazepam (w/v, in sterile deionized water). The mixture was placed in the dark for 15 min at 25 °C, after which it was analyzed for absorbance at 540 nm. The concentration of sodium nitrite (NaNO₂) was obtained using standard curves, and the results expressed as µmol·g⁻¹ Pro.

Nitric oxide synthase (NOS) activity was assayed with an NOS assay kit (NOS A014-2, Nanjing Jiancheng Biotechnology Institute, Nanjing, China). For enzyme extraction, 0.25 g samples were placed in a pre-cooled mortar (30 min at -20 °C), 4 mL of a 0.1 mol·L⁻¹ phosphate buffer (pH 7.4) were added, and the mixture was ground on ice into a homogenate. This was centrifuged at 7000 g for 15 min and served as the enzyme solution. For NOS activity, the absorbance was determined at 530 nm (light path, 1 cm) against double distilled water as the blank. The activity of NOS was calculated from the absorbance value.

Nitrate reductase (NR) activity was determined with an NR kit (NR A096, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). For enzyme extraction, each sample was mechanically homogenized with nine times the volume of the buffer (1 : 9, w/v) in an ice-water bath. The homogenate was centrifuged at 6000 g for 10 min and used as the enzyme extract. For NR activity, absorbance was determined at 540 nm (light path, 1 cm) against double distilled water as the blank. The OD of each sample was used to calculate NR activity.

Data analysis and statistics

Each treatment was repeated three times. All indexes were calculated using Microsoft Excel 2007 and differences were analysed by one-way ANOVA and Duncan's multiple comparison (Duncan, α = 0.05) using SPSS software (v21.0, SPSS Inc.). Pearson's method of skewness was used for correlation analysis. Graphs were plotted using Sigmaplot software (v12.5, SYSTAT). Indexes of SE were calculated as follows:

$$\text{Percentage of browning explants (\%)} = \frac{\text{Number of browning explants}}{\text{Number of surviving explants}} \times 100 \quad (1)$$

$$\text{Somatic embryogenesis percentage (\%)} = \frac{\text{Number of explant with somatic embryos}}{\text{Number of surviving explants}} \times 100 \quad (2)$$

$$\text{Number of somatic embryos per explant} = \frac{\text{Total number of somatic embryos}}{\text{Number of explants with somatic embryos}} \quad (3)$$

Results

Effect of high concentration of sucrose and PGRs on browning and somatic embryogenesis

The sucrose and PGRs treatments affected explant browning and somatic embryogenesis (Fig. 1b–h). Higher concentrations of sucrose promoted explant browning. The percentage of browning explants differed significantly among the treatments by day 60 (Fig. 2a). The percentage on medium containing 75 g·L⁻¹ sucrose and no PGRs was 94.2%, 112.2% higher than on medium containing 20 g·L⁻¹ sucrose and no PGRs. The presence of PGRs increased the percentage of browning explants. The percentage of browning explants was 100% on medium containing 75 g·L⁻¹ sucrose + PGRs, and 46.1% on medium containing 20 g·L⁻¹ sucrose + PGRs.

No somatic embryos formed from explants on media without PGRs. In the medium containing 75 g·L⁻¹ sucrose + PGRs (Fig. 2b), SE was 58.6%, more than five times higher than on medium containing 20 g·L⁻¹ sucrose + PGRs (11.4%). The number of somatic embryos was higher in explants on medium containing 75 g·L⁻¹ sucrose (20.0 somatic embryos per explant) than in explants on medium containing 20 g·L⁻¹ sucrose (14.6 somatic embryos per explant) (Fig. 2c).

Effect of high concentrations of sucrose and PGRs on programmed cell death

The effect of sucrose on mitochondrial structure was not influenced by PGRs in the medium (Fig. 3). Before culturing, cotyledon cells covered with fat granules were arranged in a regular pattern (Fig. 3a). However, after 13 days of culturing chromatin condensation had occurred inside the nuclei of explant cells on media containing 75 g·L⁻¹ sucrose (with or without PGRs). The mitochondria had a blurred structure and spherical shape (Fig. 3b and d). In contrast, the morphology and structure of mitochondria were normal in explant cells on media containing 20 g·L⁻¹ sucrose (with or without PGRs) (Fig. 3c and e).

Sucrose and the presence of PGRs in the culture medium significantly affected mitochondrial function (Fig. 4) and intracellular caspase-3-like protease activity (Fig. 5). As the culturing increased, mitochondrial membrane absorbance (Fig. 4a), membrane potential (Fig. 4b), and mitochondrial Cyt *c/a* (Fig. 4c) decreased ($P_{9d} < 0.05$, $P_{11d-13d} < 0.01$), while caspase-3-like protease activity increased ($P_{5d-11d} < 0.05$, $P_{13d} < 0.01$, Fig. 5). When explants were on the high sucrose concentration medium, mitochondrial membrane potential and absorbance decreased, while caspase-3-like protease activity increased during the culturing period. For a given sucrose concentration, mitochondrial

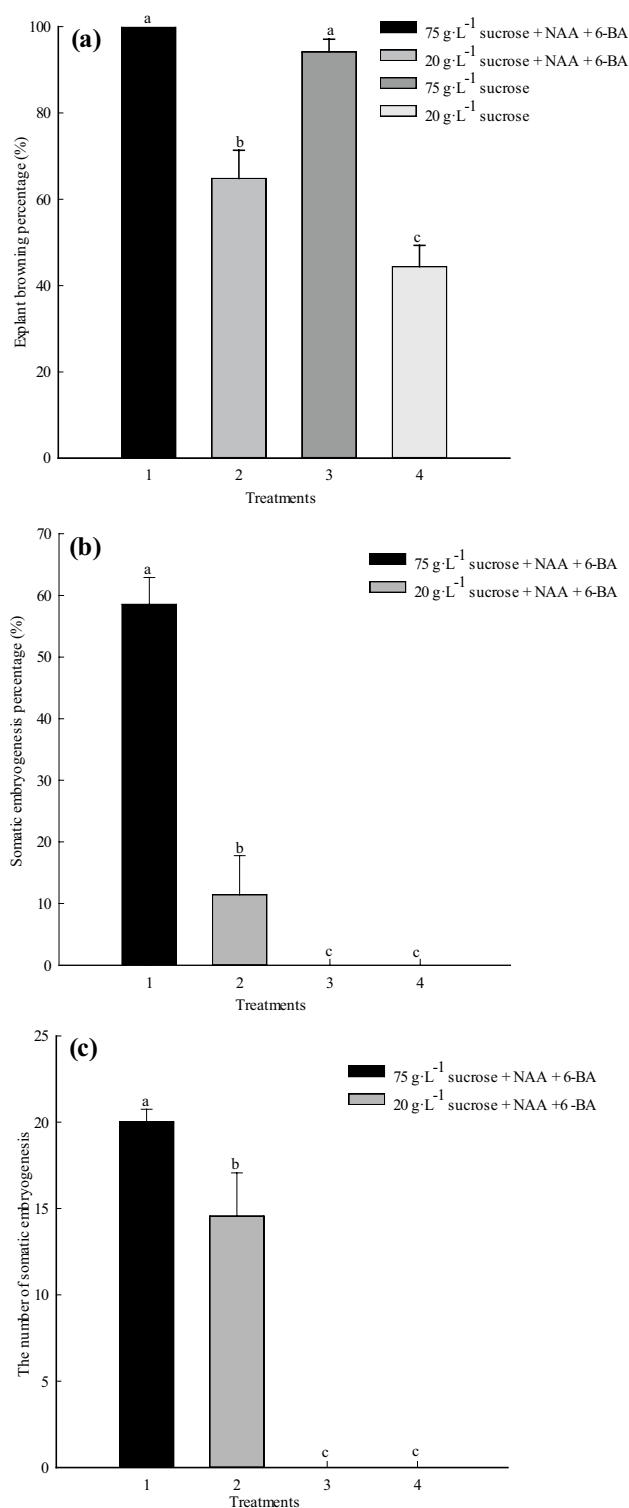
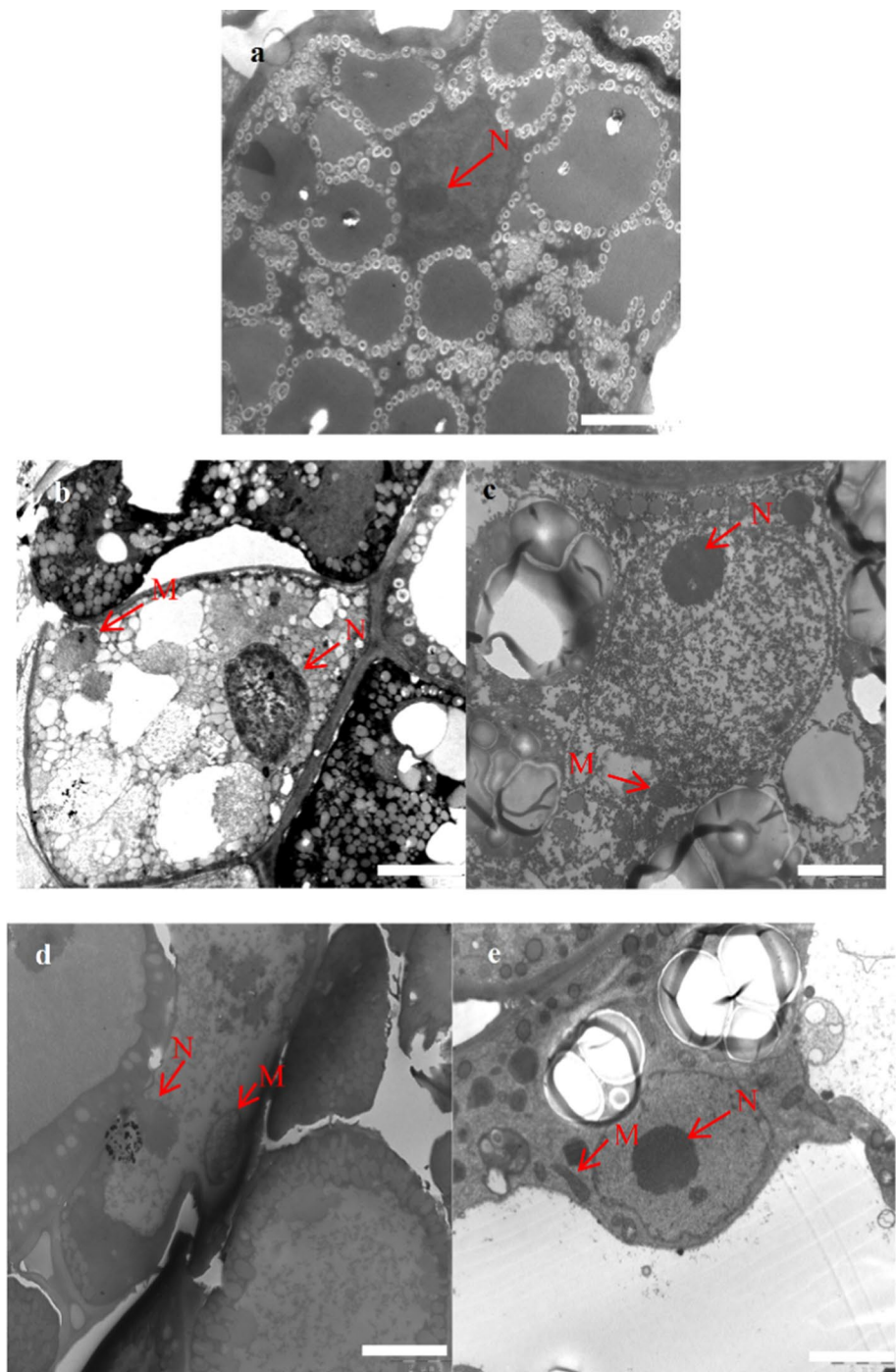


Fig. 2 Somatic embryogenesis and explant browning in *Fraxinus mandshurica*. **a** explant browning percentage; **b** somatic embryogenesis percentage; **c** number of somatic embryos. Note: Treatments with different letters are significantly different at $P=0.05$ using Duncan's Multiple Range Test

Fig. 3 Changes in mitochondria and nuclei in cells of *Fraxinus mandshurica* by day 13 of culture. **a** Explant cells before culture; **b** Explants in medium with $75 \text{ g}\cdot\text{L}^{-1}$ sucrose + $26.84 \mu\text{M}$ NAA + $8.88 \mu\text{M}$ BA after 13 days in culture. Chromatin condensates inside the nucleus and the internal structure of mitochondria become blurred and shows a spherical shape; **c** Explants in medium with $20 \text{ g}\cdot\text{L}^{-1}$ sucrose + $26.84 \mu\text{M}$ NAA + $8.88 \mu\text{M}$ BA after 13 days in culture; **d** Explants on medium with $75 \text{ g}\cdot\text{L}^{-1}$ sucrose after 13 days in culture; **e** Explants on medium with $20 \text{ g}\cdot\text{L}^{-1}$ sucrose after 13 days in culture. Mitochondrial morphology is normal and nuclear chromatin is uniform. Bar = $2 \mu\text{m}$ (a–e). *M* Mitochondrion; *N* Nucleus



membrane absorbance and potential were lower, and caspase-3-like protease activity higher if PGRs were present.

Effect of high concentrations of sucrose and PGRs on H_2O_2 metabolism

High concentrations of sucrose and PGRs increased H_2O_2 level in explants. As culturing time extended, intracellular H_2O_2 levels increased in all treatments ($P_{5\text{d}-13 \text{d}} < 0.01$,

Fig. 6a). At various times, the lowest intracellular H_2O_2 level was in explants on medium containing $20 \text{ g}\cdot\text{L}^{-1}$ sucrose and no PGRs, while the highest was in explants on medium containing $75 \text{ g}\cdot\text{L}^{-1}$ sucrose + PGRs.

High concentrations of sucrose increased SOD activity while PGRs had the opposite effect. The highest SOD activity was in explants on medium containing $75 \text{ g}\cdot\text{L}^{-1}$ sucrose and no PGRs, except on day 11. During the culturing period, SOD activity first increased and then decreased in all

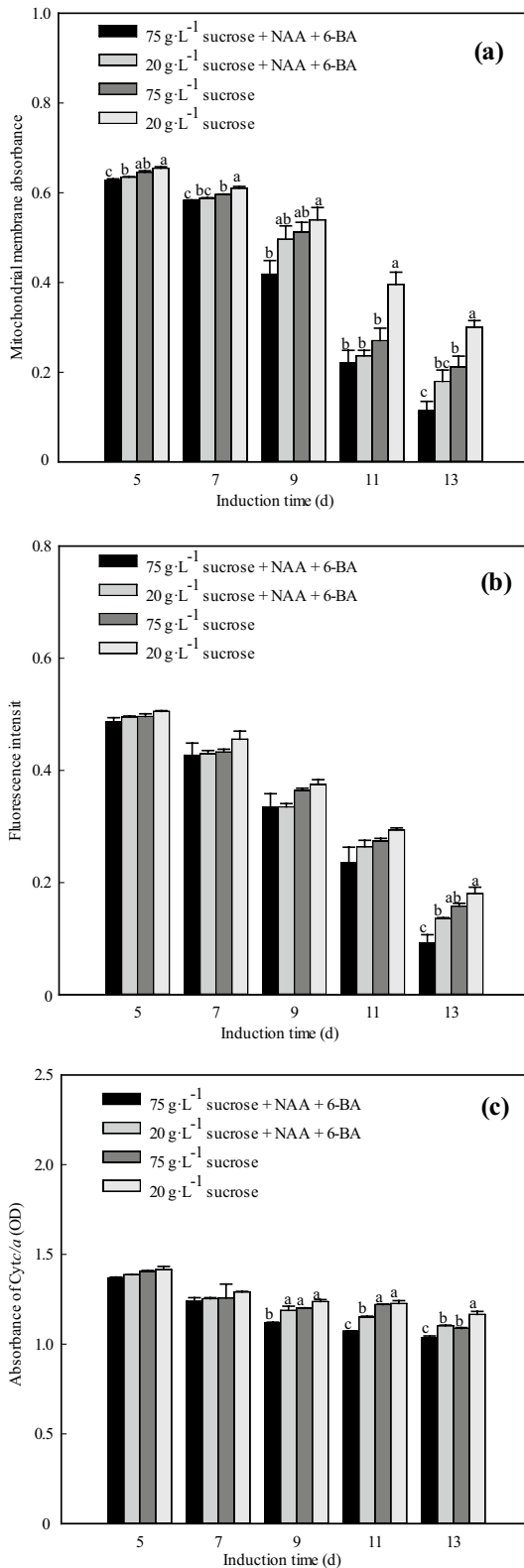


Fig. 4 Effect of different treatments on mitochondrial function index in *Fraxinus mandshurica*. **a** Mitochondrial membrane absorbance; **b** Mitochondrial membrane potential; **c** Cyt *c/a*. Note: Treatments with different letters are significantly different at $P=0.05$, using Duncan's Multiple Range Test

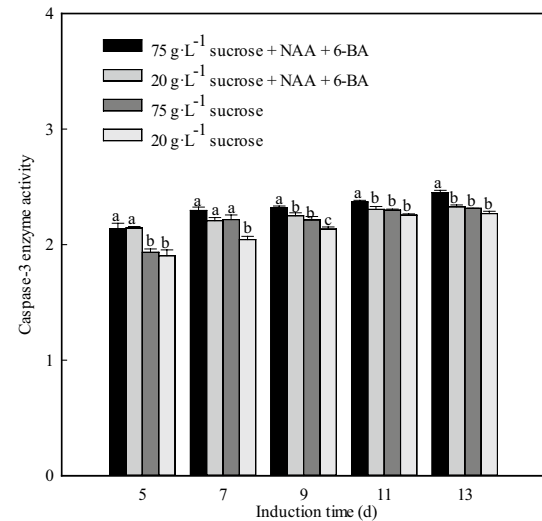


Fig. 5 Effect of different treatments on intracellular caspase-3-like protease activity in *Fraxinus mandshurica*. Note: Different letters are significantly different from each other at $P=0.05$, using Duncan's Multiple Range Test

treatments ($P_{5d} > 0.05$, $P_{7d-11d} < 0.05$, $P_{13d} < 0.01$, Fig. 6b), whereas POD activity increased continuously until the end of the culturing period ($P_{5d} > 0.05$, $P_{7d-13d} < 0.01$, Fig. 6c). For explants on medium containing 20 g·L⁻¹ sucrose and no PGRs, SOD activity increase was delayed, reaching a maximum on day 11 (compared with day 9 in the other three treatments) (Fig. 6b).

Both concentrations of sucrose and PGRs promoted intracellular POD activity, which reached a maximum level (0.832 unit·g⁻¹ Pro·min⁻¹) on medium containing 75 g·L⁻¹ sucrose + PGRs by day 13 (Fig. 6c). This activity was markedly higher than in the other treatments; the lowest POD activity (0.366 unit·g⁻¹ Pro·min⁻¹) was in explants on medium containing 20 g·L⁻¹ sucrose and no PGRs.

Both concentrations of sucrose and PGRs inhibited intracellular CAT activity in explants. As culturing time extended, CAT activity first increased, then decreased, and then increased again, but the timing of the fluctuations varied among the treatments (Fig. 6d). The first peak in CAT activity was on day 9 in explants on medium containing 75 g·L⁻¹ sucrose + PGRs, two days later than in the other treatments. On day 13, CAT activity increased to 1.39 mg H₂O₂·g⁻¹ Pro·min⁻¹ in explants on medium containing 20 g·L⁻¹ sucrose and no PGRs. This was significantly higher than in the other treatments and 105.7% higher than the lowest CAT activity (0.68 mg H₂O₂·g⁻¹ Pro·min⁻¹) in explants on medium containing 75 g·L⁻¹ sucrose + PGRs.

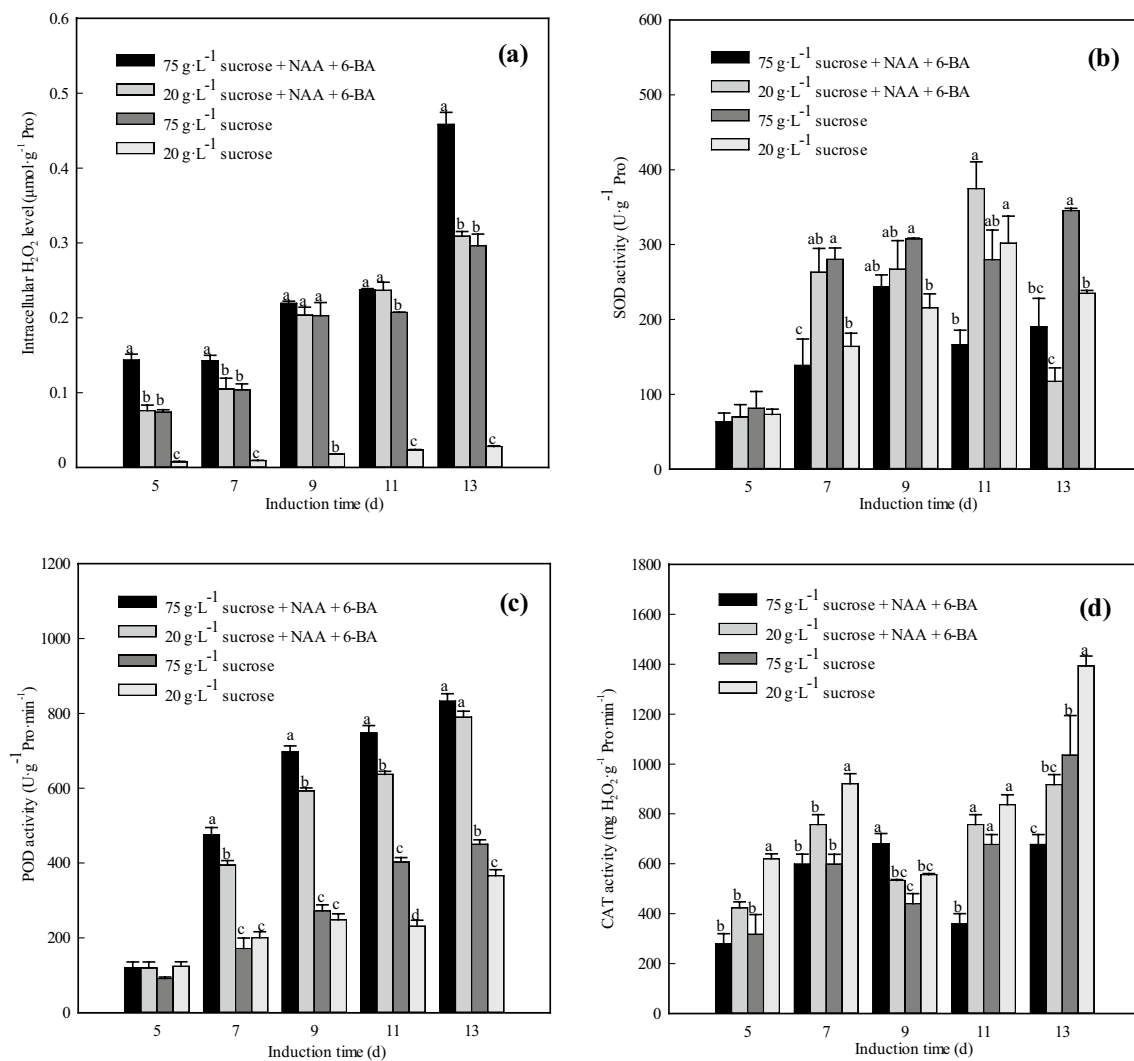


Fig. 6 Effect of different treatments on intracellular H₂O₂ and anti-oxidase in *Fraxinus mandshurica*. **a** H₂O₂, **b** SOD, **c** POD, **d** CAT. Note: Different letters are significantly different from each other at $P=0.05$, using Duncan's Multiple Range Test

Effect of high concentrations of sucrose and PGRs on enzymes related to NO synthesis

Effects of sucrose levels and PGRs on intracellular NO synthesis did not show a distinct regular pattern. As the culture time extended, nitric oxide first increased and then decreased in all treatments, but the timing of peak NO levels differed among treatments (Fig. 7a).

High concentrations of sucrose and PGRs inhibited NOS. As culturing time extended, NOS activity increased and then decreased in all treatments. NOS activity was significantly higher in explants on medium with 20 g·L⁻¹ sucrose and no PGRs than in explants on the other media (Fig. 7b). The highest NOS activity (4.4 unit·mg⁻¹ Pro) was on day 13 of culturing on medium containing 20 g·L⁻¹ sucrose and no PGRs. This was 3.4 times higher than that

in explants on medium with 75 g·L⁻¹ sucrose + PGRs (1.30 unit·mg⁻¹ Pro).

Sucrose concentrations and PGRs also affected NR activity. As culturing time increased, NR activity increased in explants on medium containing 20 g·L⁻¹ sucrose and no PGRs. NR activity increased and then decreased in the other three treatments but the time of peak activity differed among the treatments (Fig. 7c). The peak in NR was on day 7 in explants on medium containing 75 g·L⁻¹ sucrose and no PGRs, on day 9 on medium containing 75 g·L⁻¹ sucrose + PGRs, on medium containing 20 g·L⁻¹ sucrose + PGRs, and on day 13 on medium containing 20 g·L⁻¹ sucrose and no PGRs (Fig. 7c).

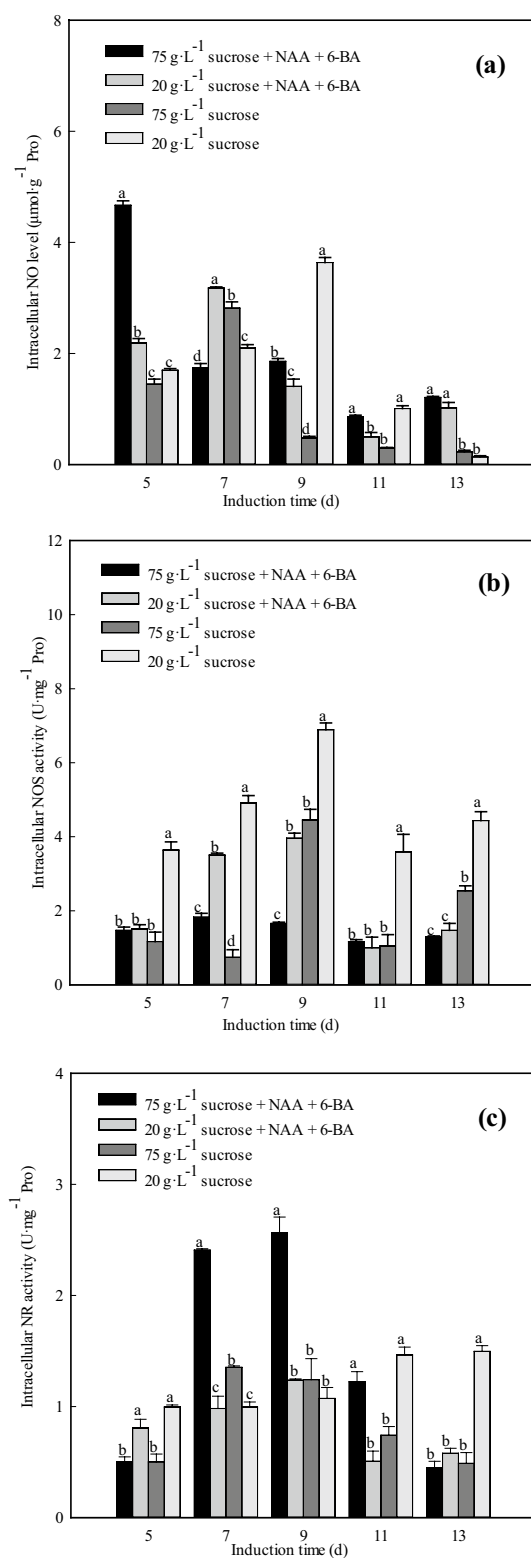


Fig. 7 Effect of different treatments on intracellular NO and related synthetic enzymes in *Fraxinus mandshurica*. **a** NO; **b** Nitric oxide synthase (NOS); **c** Nitrate reductase (NR). Note: Different letters are significantly different from each other at $p=0.05$, using Duncan's Multiple Range Test

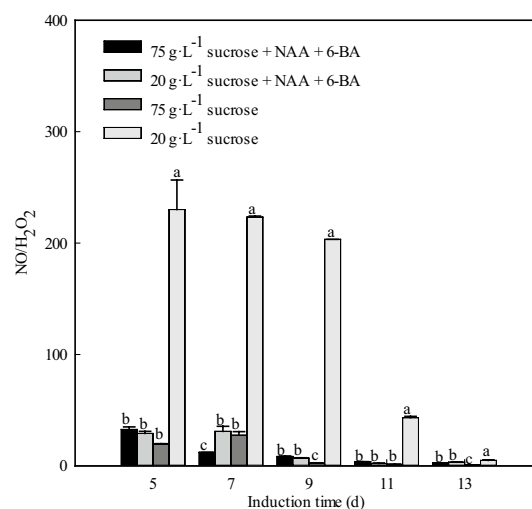


Fig. 8 Effect of different treatments on intracellular NO/H₂O₂ in *Fraxinus mandshurica*. Note: Different letters are significantly different from each other at $P=0.05$, using Duncan's Multiple Range Test

Effect of high concentrations of sucrose and PGRs on NO/H₂O₂ ratios

The ratio of NO to H₂O₂ was reduced by high sucrose levels and PGR treatments and was consistently higher in explants treated with 20 g·L⁻¹ sucrose and no PGRs than in the other treatments. As the culturing time extended, NO/H₂O₂ ratios decreased in all treatments ($P_{5d} < 0.05$, $P_{7d-13d} < 0.01$, Fig. 8). By day 5, the highest NO/H₂O₂ ratio (230.1) was in explants on medium containing 20 g·L⁻¹ sucrose and no PGRs, and was significantly higher than those in other treatments. The lowest NO/H₂O₂ ratio (19.6) was in explants on medium containing 75 g·L⁻¹ sucrose and no PGRs.

Discussion

High sucrose concentrations promote somatic embryogenesis induced by plant growth regulators

Plant growth regulators are necessary for SE in species such as *F. mandshurica* and *F. excelsior* (Capuana et al. 2007). Our results also show that high sucrose concentrations can promote SE in explants of *F. mandshurica*. This is consistent with the results of Eapen and George (1993) for peanut (*Arachis hypogaea* L.). Similarly, Krishnan and Siril (2017) suggested that prolonged nutritional stress along with high levels of exogenous auxins might lead to a higher percentage of embryo induction from callus tissues. The presence of auxin in the medium may affect gene expression in differentiating cells of *Arabidopsis* by increasing demethylation of

DNA (Grzybkowska et al. 2018). They reported a decrease in the global DNA methylation levels during SE that contrasted with the up-regulation of METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3) DNA methylases and the down-regulation of DNA demethylases, including REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), and DEMETER-LIKE 2 (DML2).

High sucrose and PGRs concentrations promote programmed cell death during somatic embryogenesis

Yang et al. (2019) detected DNA fragmentation in the nucleus of explant cells during the induction of SE in *F. mandshurica*, suggesting that somatic embryogenesis in this species is accompanied by PCD of some explant cells. Mitochondria are important organelles in plant cells. They are regulators and amplifiers of apoptotic signals and are involved in PCD (Zhao et al. 2018). Damage to mitochondrial structure often occurs in the late stages of apoptosis or cell death, and is a typical marker of PCD (Doorn et al. 2011). In this study, when cotyledons of *F. mandshurica* were cultured on medium containing 75 g·L⁻¹ sucrose for 13 days, the internal structure of the mitochondria became blurred (Fig. 3), indicative of functional damage. These results provide evidence that high concentration of sucrose causes programmed cell death in some explant cells during somatic embryogenesis of *F. mandshurica*.

Changes in mitochondrial membrane absorbance are indicative of changes in permeability (Zhan et al. 2014). In this study, the higher the sucrose concentration in the medium, the higher the mitochondrial membrane permeability. This supports the finding that PCD induced by high sucrose and PGR concentrations is dependent on mitochondrial membrane pore openings, which affects permeability (Panda et al. 2008). Membrane permeability increased and membrane potential decreased; at the same time, mitochondrial Cyt c decreased during somatic embryogenesis (this study; Zhan et al. 2014). The higher the concentration of sucrose in the medium, the lower the Cyt *c/a* ratio, and the presence of PGRs increased the magnitude of this reduction. Thus, the results show that mitochondrial membrane structure and function are seriously damaged during PCD induced by high concentration of sucrose and PGRs.

The results also show that caspase-like protease is involved in the process of programmed cell death in somatic embryogenesis of *F. mandshurica*. In this study, the highest caspase-3-like protease activity was in explants on medium containing 75 g·L⁻¹ sucrose + PGRs (NAA + BA), and the activation of enzyme activity was also faster in this treatment than in the other treatments (Fig. 5). This is consistent with the results of Biswas and Mano (2016) who reported that lipid peroxide-derived reactive carbonyl species (RCS),

which are downstream products of ROS, mediate oxidative signals to initiate PCD in plants.

H₂O₂ in the induction of PCD during somatic embryogenesis

The results show that H₂O₂ plays an important role in somatic embryogenesis of *F. mandshurica*. As culturing time extended, the H₂O₂ levels increased during SE. At a given culture point, the H₂O₂ was related to the rate of SE, i.e., highest in the 75 g·L⁻¹ sucrose + PGRs treatment, followed by the 20 g·L⁻¹ sucrose + PGRs treatment, the 75 g·L⁻¹ sucrose and no PGRs treatment, and lowest in the 20 g·L⁻¹ sucrose and no PGRs treatment (Figs. 2b and c, 6a). Similar results have been reported for *Gossypium hirsutum* L. (Cheng et al. 2015) and *Larix leptolepis* (Lamb.) Carr. (Zhou et al. 2016), suggesting that higher H₂O₂ induced by higher sucrose and PGRs concentrations may be a key condition for SE in plants. In addition, in the correlation analyses, H₂O₂ levels were significantly associated with SOD, POD, and CAT activity (Supplementary Tables S1, S2, S3, and S4). Its metabolism was closely related to SE in *F. mandshurica*. This result is consistent with observations in other woody species as reported by Yang et al. (2019) and Zhang et al. (2010). Salo et al. (2016) found that entry into the embryogenic pathway is related to cellular stress responses during somatic embryogenesis in Scots pine (*Pinus sylvestris* L.). Their results indicated that high sucrose concentration triggers the embryogenic pathway and activates a wide range of stress defence mechanisms. Thus, the manipulation of stress response pathways may provide a way to enhance SE in recalcitrant *F. mandshurica* lines.

Crosstalk between H₂O₂ and NO in the SE and PCD of explant cells

As a secondary messenger in plants, NO plays an important role in biological activities. Recent studies have explored the crosstalk between H₂O₂ and NO signals (Ahmad et al. 2019). In this study, correlation analysis showed that NO levels in explants on medium containing 75 g·L⁻¹ sucrose + PGRs were significantly correlated with the activities of antioxidant enzymes (SOD, POD, and CAT) (Table S1) but not significantly correlated with H₂O₂. This suggests that intracellular NO levels affect H₂O₂ metabolism, and the NO signal is located upstream of the H₂O₂ signal. This is similar to the results of Yao et al. (2012) but in contrast to Lum et al. (2002). In addition, Huang et al. (2014a) showed that the NO signal is located upstream of the H₂O₂ signal in the signalling network regulating haemoglobin-controlled cell survival or death during in vitro plant embryogenesis.

Both NOS and NR are potential sources of intracellular NO (Deng et al. 2016). The activities of these two enzymes

were monitored during somatic embryogenesis and there were no significant correlations between intracellular NO content and NOS or NR activities (Tables S1, S2, S3 and S4). Instead, there may be non-enzymatic reactions related to NO synthesis during the SE process (Ahmad et al. 2019).

The interaction between NO and ROS determines the occurrence of PCD (Balestrazzi et al. 2011; Wilkins et al. 2011). In this study, caspase-3-like protease activity showed a positive correlation with intracellular H₂O₂ in treatments with 75 g·L⁻¹ sucrose + PGRs and with 20 g·L⁻¹ sucrose + PGRs. Intracellular NO and NO/H₂O₂ ratios were significantly negative with caspase-3-like protease activity (Tables S1 and S2). However, NO content in explants on a medium of 75 g·L⁻¹ sucrose + PGRs was significantly higher than on 20 g·L⁻¹ sucrose + PGRs at 5, 9 and 11 days, and explant browning was significantly higher with 75 g·L⁻¹ sucrose + PGRs than with 20 g·L⁻¹ sucrose + PGRs, as well as caspase-3-like protease activity. These results indicate that H₂O₂ is a positive regulator of somatic embryogenesis in *F. mandshurica* and programmed cell death of explant cells, while the regulation of nitric oxide is complex.

In summary, plant growth regulators play a decisive role in somatic embryogenesis of *F. mandshurica*, and promoted by H₂O₂ and NO signals caused by high sucrose concentration. In addition, H₂O₂ is involved in the induction of programmed cell death in explant cells, and is related to NO synthesis. Our findings imply that H₂O₂ is a positive regulator of somatic embryogenesis in *F. mandshurica* and programmed cell death while NO regulation is complex. The manipulation of stress response pathways may provide a way to enhance somatic embryogenesis in recalcitrant *F. mandshurica* lines.

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Authors' contributions Y. L. and S. HL conceived and designed the study. Y. L. and Z. DY collected plant materials and prepared SE samples for analysis. W. H. and Z. DY analyzed the results for experiments. Y. L., Z. DY and W. H. contributed to the writing of the manuscript and data analyses. F. JL and S. HL revised the manuscript. All authors read and approved the final manuscript.

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