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Protocol

Visualisation of Reactive Oxygen Species During Stress of Aromatic Crop

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Abstract: The present protocol described staining protocol for Reactive Oxygen Species (ROS) in aromatic crop grown under nutrient stress through DAB and NBT histochemical method. Spearmint (*Mentha spicata*) were grown under manganese and salt toxicity stresses and after 10 and 20 days of the stress treatments, plants showed stunted growth. The morphological characteristics of leaves under stresses were observed. Manganese toxicity and salt stress induced the production of ROS. Accumulation of hydrogen peroxide was characterised as brown spots from the DAB polymerisation which were emerged and clearly observed in leaves from plant grown under 2.5 and 5 mM concentrations of manganese as well as 300 mM concentration of salt. Furthermore, accumulation of superoxide anion was characterised as blue pigments based upon the ability of cells to reduce NBT. Spearmint leaves showed the distribution of the blue pigment which was obviously observed under the 5 mM of manganese and 300 mM of salt. DAB and NBT staining method can be the rapid method to characterise ROS accumulation in plant cell under the abiotic stresses.

Keywords: ROS; histochemical; superoxide; hydrogen peroxide

1. Introduction

Reactive Oxygen Species (ROS) are generally recognised as potentially damaging by product of plant aerobic metabolism. The operation of photosynthesis also results in a highly increase in the production of the ROS such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$). Metabolic pathways in plant organelles are sensitive to changes in environmental conditions, and metabolic imbalances can induce an oxidative stress in cells by promoting the generation and accumulation of reactive oxygen species (ROS), causing oxidation of cellular components, hindering metabolic activities and affecting organelle integrity (Suzuki et al., 2012). The histochemical detection of ROS provides precise information about the *in situ* distribution and accumulation of ROS in different cells and tissues over a relatively large area, e.g. whole leaf blade. Routinely, 3,30-diaminobenzidine (DAB) and nitro blue tetrazolium chloride (NBT) are used as chromogens for the assessment of hydrogen peroxide and superoxide anion, respectively (Thordal-Christensen et al., 1997). This technique was successfully used as to detected plant stress under biotic stress such as in leaves of common ice plant (*Mesembryanthemum crystallinum*), pumpkin (*Cucurbita maxima*), and cucumber (*Cucumis sativus*) that were infected by fungus. By performing these methods, plant tissue showed variability in DAB/NBT staining quality and stresses produce a range of staining patterns. For visualisation of the hydrogen peroxide, the marked brown polymerisation product is formed by the reaction of DAB with hydrogen peroxide. Histochemical staining for superoxide anion in leaf

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tissue is based on the ability of cells to reduce NBT. NBT specifically reacts with superoxide and forms a purple/blue formazan precipitate (Sekulska-Nalewajko et al., 2016). These techniques have not been used in any aromatic crops. In this work we applied these histochemical procedure as to detect the ROS during green-house production of spearmint (*Mentha spicata*) with salt and manganese stresses.

2. Experimental Design

This protocol was conducted towards characterising H_2O_2 and $O_2^{\bullet-}$ in spearmint (*Mentha spicata*) leaves under nutrient stress. Plants were grown under four levels of manganese (0, 1, 2.5, and 5 mM) and three levels of salt (0, 100, and 300 mM) in glasshouse. Five fully expanded young leaves of each treatments were harvested for histochemical analysis at 10 days and 18 days after transplanting. Characterization of hydrogen peroxide and superoxide were determined by DAB and NBT staining method, respectively.

2.1. Materials

- 3, 3′ -Diaminobenzidine (DAB) (Sigma-Aldrich, China; Cat. no.: D12384)
- Nitro blue tetrazolium chloride (NBT) (Merck, Germany; Cat. no.:596470)
- Hydrochloric acid (RCI Labscan, Thailand; AR1107)
- Phosphate buffer solution, 10mM, pH 7.8
- Sodium azide (NaN_3) (Ajax Finechem, New Zealand; Cat. no.: 1222)

2.2. Equipment

- Vacuum chamber
- Vacuum pump

3. Procedure

3.1 Histochemical of hydrogen peroxide (H_2O_2) free radical

The presence of H_2O_2 in leaves was detected by DAB staining method (Sekulska-Nalewajko et al., 2016; Sunkar, 2010). Leaves were submerged in 1 mg/mL DAB–HCl (pH3.8) for 12 hours under light condition. Polymerization of DAB at the sites of H_2O_2 accumulation generates a brown DAB-polymer.

3.2 Histochemical of superoxide ($O_2^{\bullet-}$) free radical

The presence of $O_2^{\bullet-}$ in leaves was detected by NBT staining method (Sunkar, 2010; Thordal-Christensen et al., 1997). Leaves were immersed in NBT-PBS solution containing 10 mM PBS (pH 7.8), 0.1 mM NBT and 10 mM NaN_3. The solution was vacuum infiltrated for 15 minutes. The leaves were then held at room temperature until the blue spot of formazan that is produced as a result of the reduction of NBT by O_2 free radical became visible.

4. Expected Results

Two-week-old cutting of spearmint (*Mentha spicata*) was repotted into a 11×11×12 cm^3 pot filled with coarse sand: TYP ED 73 soil. The mint plants (n=120) were grown for 20 days in a glasshouse. Plants were maintained under an in-house routine maintenance, prior to treatments. Manganese salt and stress treatments were prepared in Hoagland's solution described in Table 1 with the additional of Mn to 1, 2.5, and 5 mM (Mn stress treatment) and salt to 100, and 300 mM (salt stress treatment). Treatments were applied daily (50 mL per plant) in Completely Randomised Design. Physiological morphology of plant as well as histochemical of ROS were observed.

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Table 1. Concentration ranges of nutrient elements of Hoagland 's nutrient solution (Hoagland and Arnon, 1938)

Nutrient elements	Concentration (mg L^{-1})
N	210
P	31
K	234
Ca	160
Mg	34
S	64
Fe	2.5
Cu	0.02
Zn	0.05
Mn	0.5
B	0.5
Mo	0.01

4.1 Physiological morphology

The toxic effects of many essential elements induced oxidative stress which have been linked to the increased production of ROS, such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2). Manganese is an essential micronutrient which is involved in both oxygen radical production via its involvement in the photosynthetic pathway, and oxygen radical detoxification (Hänsch and Mendel, 2009) . In Rice (*Oryza sativa* L.) cv. Pant-12, treated with 6mM of manganese showed increased generation of superoxide anion ($O_2^{\bullet-}$), elevated levels of hydrogen peroxide (H_2O_2) and imbalanced the levels of antioxidants and the antioxidative enzymes (Srivastava and Dubey, 2011). Excessive amount of salt enters the plant in the transpiration stream and it can injure leaf cell tissue and inhibit plant growth. Plants under salt stress might suffer oxidative damage gave rise of the reactive oxygen species (ROS) level. Redox signal molecules including electron carriers, electron acceptors as well as ROS act as a local or systemic signal for leaf stomata closure which lead to osmotic stress due to the imbalance of osmotic pressure (Chaves et al., 2009; Shabala, 2017).

In our work, spearmint grown under Mn toxicity (5 mM) showed stunted growth with lower number of leaves as compared to the control (0 mM Mn) (Figure 1). Leaves under manganese toxicity stress showed the necrotic brown spots which distributed over leaf blade (Figure 2). In the salt stress treatment, plant growth under salt stress was obviously inhibited as compared to control (0 mM salt). Leaves showed the lighter shade of green under 300 mM salt (Figure 3). The morphological characteristics of spearmint leaves under salt stress were severely necrotic in leave tissue from the base of the petiole and leaves became wilting due to the imbalance of osmotic pressure (Figure 4).

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Figure 1. Physiological morphology of spearmint (*Mentha spicata*) grown under four levels of manganese (0 (control), 1, 2.5, and 5 mM) for 20 days after transplanting in glasshouse.

Figure 2. Morphological characteristics of spearmint (*Mentha spicata*) leaves under manganese toxicity stress for 20 days after transplanting in glasshouse.

Figure 3. Physiological morphology of spearmint (*Mentha spicata*) grown under three levels of salt (0 (control), 100, and 300 mM) for 20 days after transplanting in glasshouse.

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Figure 4. Morphological characteristics of spearmint (*Mentha spicata*) leaves under salt stress for 20 days after transplanting in glasshouse.

4.2 Histochemical of ROS

Hydrogen peroxide accumulation was detected in spearmint (*Mentha spicata*) leaves grown under manganese toxicity and salt stress as observed in the brown spots from polymerisation of DAB. Leaves which were collected from plant grown under 2.5 and 5 mM concentrations of manganese for 18 days after transplanting (Harvest 2) showed more distribution of the brown pigments comparing to the control (0 mM Mn). However, salt stress plants which were grown under 100 mM concentration of salt for 18 days after transplanting (Harvest 2) showed the distribution of brown pigments while plants grown under 300 mM concentration of salt showed the distribution of brown pigment from 10 days after transplanting (Harvest 1) (Figure 5).

Superoxide anion accumulation was detected in spearmint (*Mentha spicata*) leaves as observed as blue colours due to the reduction of NBT. Manganese toxicity stress at both 2.5 and 5 mM illustrated the dark blue pigments from 10 to 18 days after transplanting (Harvest 1 and 2) while salt stress (100 and 300 mM salt) showed the clearly distribution of dark blue pigments at 18 days after transplanting (Harvest2). In addition, leaves from plants which were grown in 300 mM concentration of salt showed the distribution of dark blue pigments in both 10 and 18 days after transplanting (Harvest1 and 2) (Figure 6)

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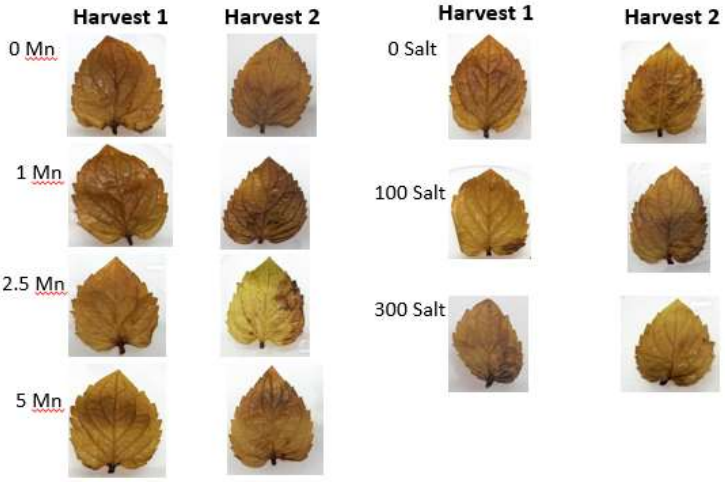

Figure 5. Detection of hydrogen peroxide accumulation in spearmint (*Mentha spicata*) leaves under three levels of and four levels of manganese (0, 1, 2.5, and 5 mM) and three levels of salt (0, 100, and 300 mM) in glasshouse at 10 days (Harvest 1) and 18 days (Harvest 2) after transplanting.

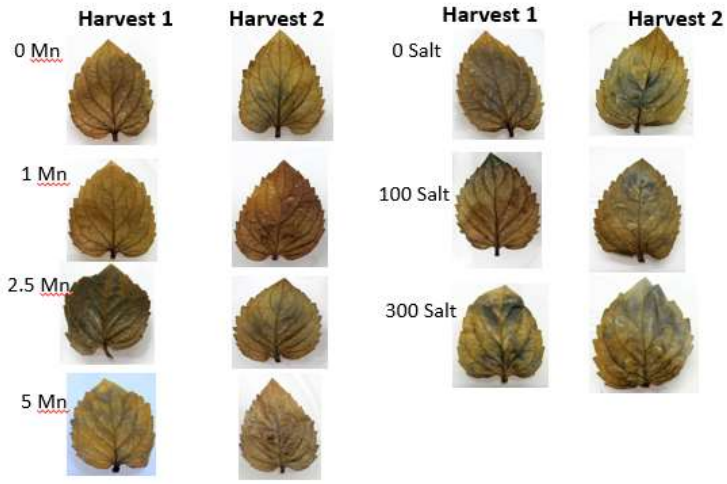

Figure 6. Detection of superoxide anion accumulation in spearmint (*Mentha spicata*) leaves under under four levels of manganese (0, 1, 2.5, and 5 mM) and three levels of salt (0, 100, and 300 mM) in glasshouse at 10 days (Harvest 1) and 18 days (Harvest 2) after transplanting.

5.Conclusion

In studying manganese toxicity and salt stress, physiological morphology could be observed along with the histochemical characteristics which showed the accumulation of hydrogen peroxide and superoxide anion represented in the distribution of DAB and NBT staining.

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Conflicts of Interest: The authors declare no conflict of interest.

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