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Localization and Identification of Phenolic Compounds in *Salvia miltiorrhiza* Bunge Roots and Leaves

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Abstract: *Salvia miltiorrhiza* Bunge is a commonly used traditional Chinese medicine whose water-soluble components are the key active ingredients for its therapeutical effects. In order to illustrate the synthesis, translocation, and distribution of the water-soluble phenolic compounds in the different growing stages of S. *miltiorrhiza* Bunge, levels of salvianolic acid B (SAB) and rosmarinic acid (RA) were examined we performed histochemistry and high performance liquid chromatography (HPLC) to quantify the amounts. We found that the main phenolic compounds synthesized initially during the sprouting stage to be SAB and RA, which are both mainly located in the central vacuoles of palisade parenchyma and epidermis cell in the leaf during this particular stage. Of the phenolic compounds initially located in the plasma membranes of periderm and phloem parenchyma cells during the germination stage of the root, there is a large amount of RA yet little presence of SAB. Located in the central vacuoles of the periderm cell of the root after leaf wilting are the main components, SAB and RA.

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Keywords: *Salvia miltiorrhiza* Bunge, Salvianolic acid B, Localization, Rosmarinic acid, Phenolic compound

1. Introduction

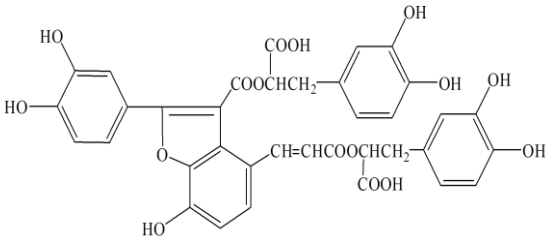
The root and rhizome of *Salvia miltiorrhiza* Bunge (*Danshen*, in Chinese) is one of the most commonly used herbal medicines in Asian countries. It has been confirmed to be effective in treating coronary heart diseases, including angina pectoris, coronary artery spasm, myocardial infarction, *etc*., based on modern investigations that reveal the herb to improve microcirculation in the human body [1-7]. The major active elements of this herb could be classified as lipid-soluble and water-soluble. The lipid-soluble components are mainly tanshinones (diterpenoid quinones) while the water-soluble components are mainly phenolic compounds [8-10]. Previous studies have shown that bioactive lipid-soluble and water-soluble components existed not only in the roots of *S. miltiorrhiza* Bunge, but also in its leaves. It is believed that a majority of the components found in the leaves are mainly water-soluble phenolic compounds [11,12].

Salvianolic acid B (SAB), one of the phenolic compounds, is the most abundant and bioactive in the roots and leaves of *S. miltiorrhiza* Bunge [13]. SAB exhibits antithrombotic effects and strong anti-oxidant properties almost a thousand times that obtained from vitamin E [14]. It has been assigned as a marker compound for *S. miltiorrhiza* Bunge in the 2005 edition of Chinese Pharmacopoeia [1]. Rosmarinic acid (RA), another important phenolic compound present in *S. miltiorrhiza* Bunge, has been reported to occur in several taxonomically non-related families of the plant kingdom and is known for ability to act as an adstringent, anti-oxidative, anti-inflammatory, anti-mutagen, anti-bacterial and anti-viral substance[15,16].

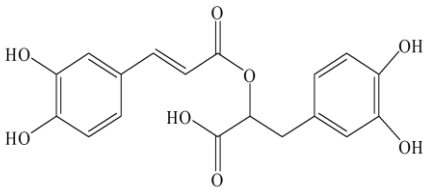
It is important to identify the dynamic profile of the concentration of water-soluble phenolic compounds at the cellular level and ascertain the location of production and storage of the phenolic components during the entire growing period. SAB and RA have been assigned as markers for water-soluble phenolic compounds. The chemical structures of these two molecules are shown in Fig.1.

In the present study, *S. miltiorrhiza* Bunge with sand culture were used as starting materials and histochemistry and HPLC were carried out to illustrate

the synthesis, translocation, and localization of phenolic compounds in different organs at various growth stages of *S. miltiorrhiza* Bunge. This present work would provide scientific basis for improving phenolic compound extractions and regulating the metabolic activity of *S. miltiorrhiza* Bunge.



SAB



RA

Fig.1. Chemical structure of SAB and RA

2. Materials and Methods

2.1 Preparation of Danshen

Danshen (*Salvia miltiorrhiza* Bunge) seeds were initially planted in sandy soil (sand: soil = 3:1). After germinating, 3–3.5cm middle roots and leaves of the plantlet growing in different days were collected, and then washed carefully for the transmission electron microscopy. Samples for High Performance Liquid Chromatography (HPLC) analysis were collected every 15-day after germinating. Before HPLC analysis, the samples were dried in vacuum oven (35 ℃), and then ground, stored in a refrigerator with the protection of nitrogen.

2.2 Histochemical localization of phenolic compounds

Histochemical localization of phenolic compounds was carried out using fresh leaves and roots at the different growth stage. Rectangular segments (1mm×2mm) of leaves were cut between the third and the fourth lateral vein, 5mm from the chief vein. The segments and middle roots were fixed for 2h in 3% glutaraldehyde (pH 7.2), prepared in 0.1M cacodylate buffer with the addition of 0.5% caffeine, and after several washes with cacodylate buffer, the specimens were post-fixed for 1.5h in buffered 1% OsO_4 at 4 ℃, as described earlier [17-20]. Caffeine was added for precipitation of phenolic compounds [21]. After fixation and dehydrated through an acetone series and embedded in Epon 812. Ultra-thin sections were stained with uranyl acetate and lead citrate and then examined with a JEOL-JEM-1230 transmission electron microscope.

2.3 HPLC analysis of phenolic compounds

Qualitative determination of phenolic compound was performed with a high-performance liquid chromatography system consisting of LC–10ATvp HPLC pump and SPD–10Avp UV-detector (Shimadzu, Japan), and the column was 5 μm Shim Park C18 (150 mm×4. 6 mm). The injection volume was 10 μl, the constant flow rate was 1ml min-1, and the detection wavelength was 281nm. Analyses were made at column temperature 30 ℃. Samples were filtered before analysis by 0.45μm millipore filter. HPLC program of phenolic compound was as described earlier [22-24]. The separation was performed by a linear elution with solvent-A (methanol) and solvent-B (2% glacial acetic acid), and mixture of elution are given as A/B (37:63, by vol.) during 0-20min.

Quantification of SAB and identification of RA were performed with standard agent, and the standards were purchased from National institute for the control of pharmaceutical and biological products in China. Quantification was based on the standard curve established with 100, 200, 300, 400 and 500 mg L-1 of SAB prepared in methanol and the results are expressed as milligrams per gram (mg/g) of dry leaves or roots weight. Phenolic compound in the dried leaves (0.1g) and roots (0.1g) were extracted by ultrasonic for 30min with 5ml 50% acquired ethanol at 25 ℃ [13,25].

3. Results

3.1 Phenolic compound distribution in leaf tissues

Epidermis, spongy parenchyma cells and palisade parenchyma cells of *S. miltiorrhiza* Bunge leaves

(Fig.2A-F and Fig.3A-G) for the different growing days contained large central vacuoles. The leaves during the 4-day growing sprout stage (Fig.2A-C) had large phenolic deposits located in the central vacuoles of palisade parenchyma cells (Fig.2A), and smaller deposits could be found along the plasma membranes (Fig.2B) and in epidermis cells (Fig.2C). The leaves in the 12-day growing stage (Fig.2D-F), viz. two-leaf one sprout stage, had no phenolic deposits observed in the palisade parenchyma cells, spongy parenchyma cells (Fig.2D and E), and vacuoles. Many phenolic aggregations associated with the plasmalemma of the epidermis (Fig.2F). The leaves in the 120-day growing stage (Fig.3A-D) entered an exuberant developmental phase of *S. miltiorrhiza* Bunge [7] and the leaf tissues had developed maturity. Large phenolic aggregations associated with the plasmalemma of the epidermis and regularly formed a thick line (Fig.3A and B). Fig.3A shows an image of a large central vacuole atrophia. No phenolic deposits were found in palisade parenchyma cells, spongy parenchyma cells, or in the central vacuoles (Fig.3C). The leaves began wilting by the 208-day growing stage (Fig.3D-F) at the beginning of November. Small phenolic compounds associated with the plasmalemma of the epidermis and other small phenolic deposits were found in epidermal vacuoles (Fig.3D and E), though no phenolic deposits were observed in other cells of the leaves (Fig.3F).

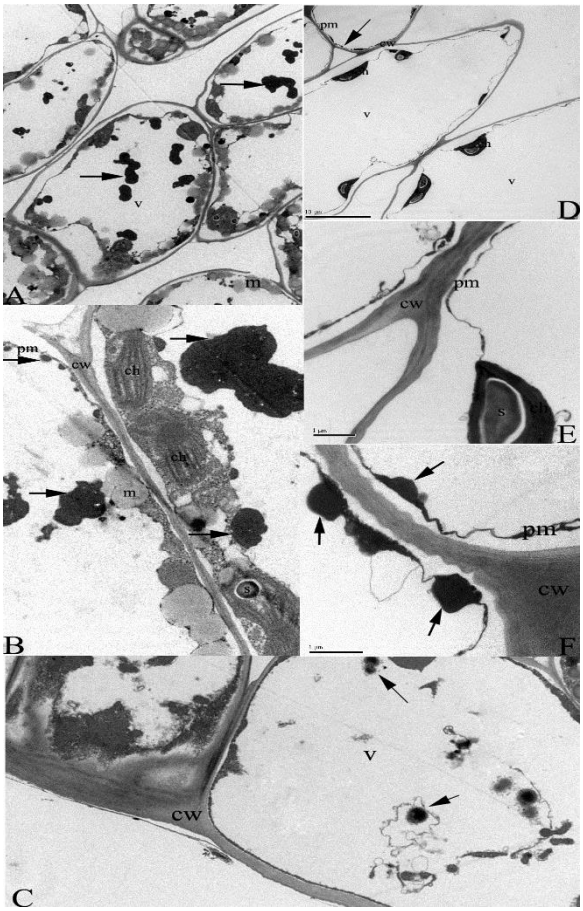


Fig.2. Ultrastructure of epidermis (C and F), palisade parenchyma (A, B and E) cells and spongy parenchyma (D) cells in leaves. Arrow: phenolic deposits; v: central vacuole; ch: chloroplasts; m: mitochondrion; pm: plasmalemma; s: grain of starch; cw: cell wall. (A, B, and C) Leaves of sprout stage, Bar = 10 μm; Bar = 2 μm; Bar = 5 μm. (D, E, and F) Leaves of two-leaf one sprout stage, Bar = 10 μm; Bar = 1 μm; Bar = 1 μm.

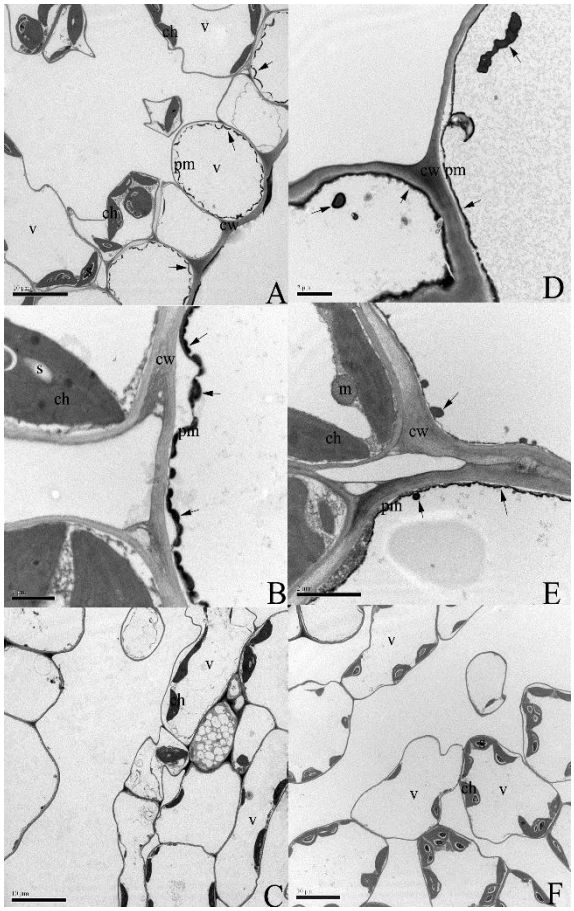


Fig.3. Ultrastructure of epidermis (A, B, D and E), palisade parenchyma (A, B and F) cells and spongy parenchyma (C and F) cells in leaves. Arrow: phenolic deposits; v: central vacuole; ch: chloroplasts; m: mitochondrion; pm: plasmalemma; s: grain of starch; cw: cell wall. (A, B, and C) Leaves of growth for 120-day after germination, Bar = 10 μm; Bar = 1 μm; Bar = 10 μm. (D, E, and F) Leaves of growth for 208-day after germination, Bar = 2 μm; Bar = 2 μm; Bar = 10 μm.

3.2 Phenolic compound distribution in root tissues

Periderm and phloem parenchyma cells of *S. miltiorrhiza* Bunge roots (Fig.4A-H and Fig.5A-G) in the different growing days contained large central vacuoles. In the germinating stage, the root tissues developed immaturely although small phenolic deposits can be seen in the plasma membranes of periderm cells and phloem parenchyma cells (Fig.4A and B). During the sprout stage, small phenolic deposits were observed in the central vacuoles of the periderm (Fig.4D). In the 12-day growing stage (i.e. two-leaf one sprout stage), many phenolic aggregations associated with the plasmalemma of periderm cell and small phenolic deposits were also found in the vacuoles (Fig.4E-H). After 120 days, the roots entered their exuberant developmental phase and subsequently developed maturity. Fig.5A shows large central vacuole and dead tissue. Massive phenolic aggregations were located along the plasma membranes of cork cambium of periderm cells (Fig.5A-C), and small phenolic deposits could be seen in the vacuoles. After 208 days, periderm cells of the roots showed many layered cells, numerous phenolic deposits were found in the vacuole of periderm cells (Fig.5D-G), and no phenolic deposits were observed in other cells of the roots.

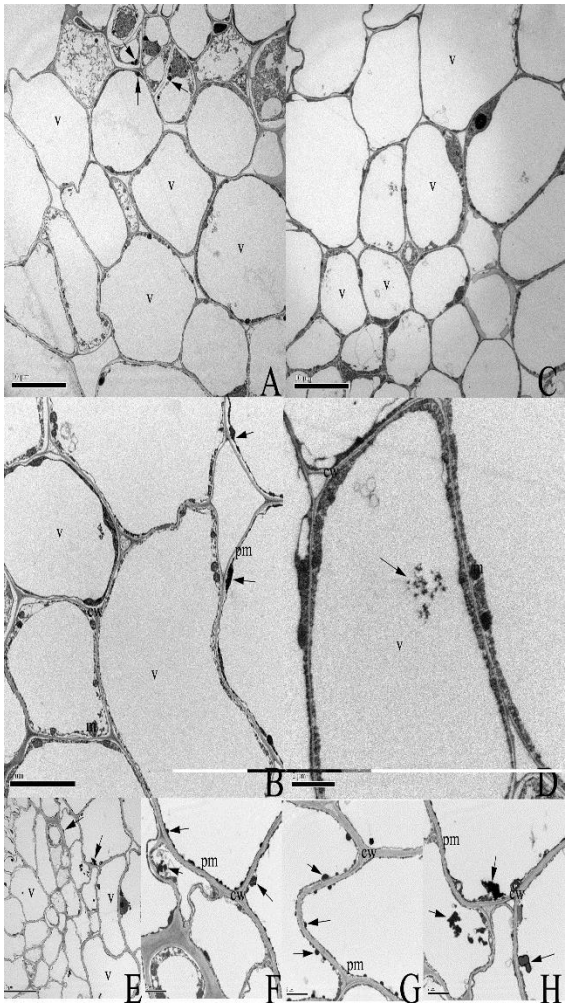


Fig.4. Ultrastructure of periderm (B, D and E-H) and phloem parenchyma cells (A, C and E) in roots. Arrow: phenolic deposits; v: central vacuole; m: mitochondrion; pm: plasmalemma; cw: cell wall. (A and B) Roots in germination stage, Bar = 10 um; Bar = 5um. (C and D) Roots in sprout stage, Bar = 10um; Bar = 5um. (E, F, G, and H) Roots in two-leaf one sprout stage, Bar = 10um; Bar = 2um; Bar = 2um; Bar = 2um.

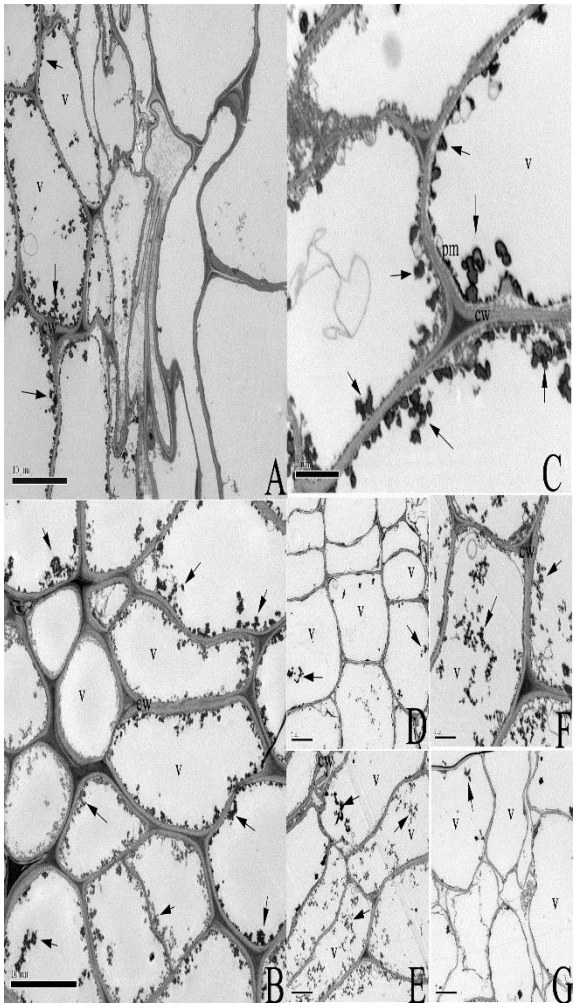


Fig.5. Ultrastructure of periderm cells (A-G) in roots of growth for 120 and 208 days after germination. Arrow: phenolic deposits; v: central vacuole; m: mitochondrion; pm: plasmalemma; cw: cell wall. (A, B, and C) Roots of growth for 120-day after germination, Bar = 10 um; Bar = 10um; Bar = 2um. (D, E, F, and G) Roots of growth for 208-day after germination, Bar = 5um; Bar = 5um; Bar = 2um; Bar = 5um.

3.3 Identification of phenolic compounds

All leaves of *S. miltiorrhiza* Bunge contained SAB and RA in the four stages (Fig.6). The contents of SAB which formed during the 4-day growth period, according to the standard curve (Y=3.27513×10-8X+0.00260287, R=0.9992), changed with plant growth and the contents in the leaves for different stages were as follows: the contents of sprout stage was 0.1256%, of two-leaf one sprout stage, 0.01697%, of growth for 120-day after germinating, 0.7181%, and for 208-day after germinating was 0.02453%. The results showed that the trend of the content for different growth stages formed an "S" curve. SAB did not appear in the roots during the germinating stage (Fig.7C) but appeared in the roots once the leaves became present (Fig.7D). When the seeds germinated into roots, we can observe the peak of RA in the chart of HPLC of the root (Fig.7C). Therefore, the RA appears earlier than SAB in the root and its occurrence increases with the growth of the root. Although the contents change with plant growth, it does not disappear until leaf wilting. The contents of SAB in the roots for different stages were as below: the content of sprout stage was 0.001726%, of two-leaf one sprout stage, 0.008175%; the content of growth for 120-day after germinating was 1.062%, for 208-day after germinating, 0.07171%. The trend of SAB content in the roots for different growth stages formed a parabolic curve.

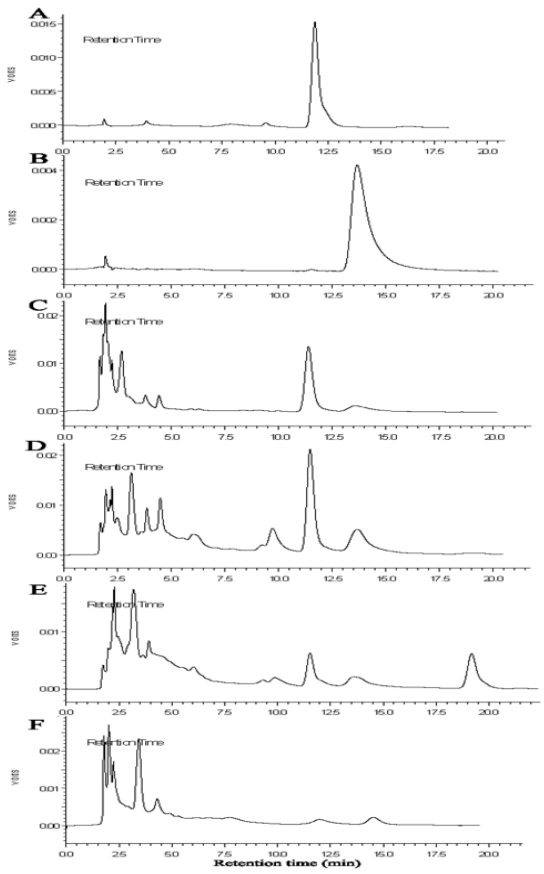


Fig.6. Chart of HPLC in leaves of different growth days

(A) HPLC chart of the standard of RA. (B) HPLC chart of the standard of SAB. (C) HPLC chart of leaves in sprout stage. (D) HPLC chart of leaves in two-leaf one sprout stage. (E) HPLC chart of growth for 120 days after germination leaves. (F) HPLC chart of growth for 208 days after germination leaves.

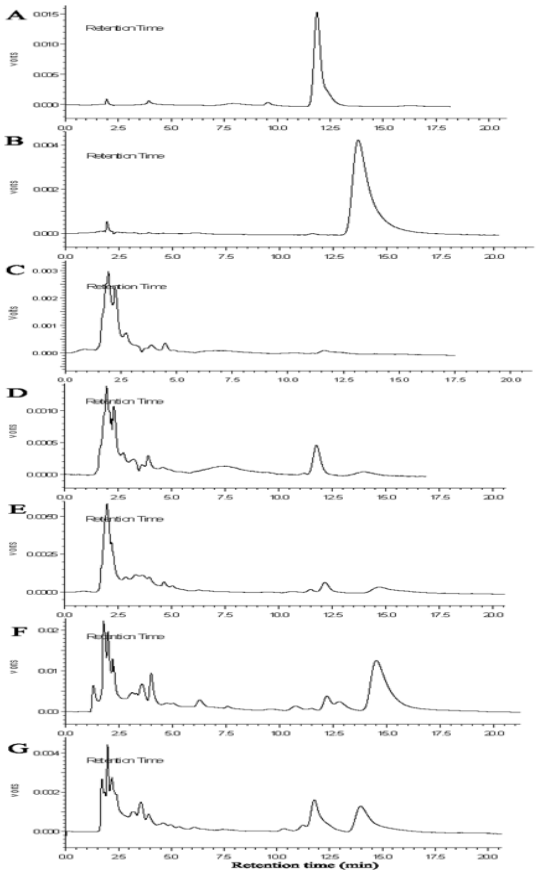


Fig.7. Chart of HPLC in roots of different growth days

(A) HPLC chart of the standard of RA. (B) HPLC chart of the standard of SAB. (C) HPLC chart of roots in germination stage. (D) HPLC chart of roots in sprout stage. (E) HPLC chart of roots in two-leaf one sprout stage. (F) HPLC chart of growth for 120 days after germination roots. (G)HPLC chart of growth for 208 days after germination roots.

4. Discussion and Conclusions

The current results showed that localization of phenolic components in *S. miltiorrhiza* Bunge roots and leaves corresponding with the growth stages, as previously reported in winter oilseed rape [19]. We speculated that different localization of phenolic components (SAB and RA) in the cell of the different growing ages was correlated with different metabolism of those cells.

Localization of phenolic components in the leaves changed with the plant growth stage. The phenolic components were present in the central vacuoles of palisade parenchyma cells and epidermis cells in the leaves during the sprout stage. From 12-day leaves (two-leaf one sprout stage) to the wilting leaves, phenolic components associated with the plasma membranes of the epidermis and were located in the central vacuoles of epidermis cells. Over the plant's growth period, the localization did not change. Such localization was in accordance with subcellular sites of phenylpropanoid biosynthesis [26]. It was also in accordance with the proposed role of SAB and RA as powerful antioxidants [16,27-29]. We observed the peaks of SAB and RA in HPLC charts of leaves from the sprout stage to the wilting leaves (Fig.6C-F) and show that phenolic components in all life stages of the leaves include SAB and RA.

Localization of phenolic components in the roots also changed with the plant growth stage. Phenolic components initially appeared in the germination stage (Fig.4B), but the main ingredient is RA and not SAB (Fig.7C). SAB initially appeared during the 4-day growth roots after germination (the sprout stage) but the RA presents in all stages of the root from the germination stage to the wilting leaves (Fig.7D-G). Phenolic components initially located in the plasma membranes of periderm cells and phloem parenchyma cells of the roots; after 120-day growth it was mainly located along the plasma membranes of cork cambium of periderm cell, and finally located in the plasma membranes and central vacuoles of periderm cell of the roots after the leaves wilting. These results were similar with the results from Brassica napus [17]. The phenolic compounds precipitated with caffeine that was found mainly in the outermost layer of the root of *Brassica napus* [30].

According to HPLC detection (Fig.6 and 7), SAB initially was synthesized in the leaves at the sprout stage. Since the SAB only occurred in the roots of the plantlet when the leaf appeared, and the content of SAB in the roots gradually reduced with the wilting leaves based on HPLC data, we speculated that SAB transported simultaneously from the leaf to the root through the stem after its synthesis.

In summary, the present observations indicated localization of phenolic compounds in the roots and leaves for the different growing ages, and the trafficking trends were clear in the roots and leaves. How the SAB is synthesized in the roots and leaves, and why the RA appears first during the germination stage, are additional questions that need to be more thoroughly investigated.

Acknowledgements

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