Pak. J. Bot., 46(5): 1847-1857, 2014.

STRUCTURE AND CHEMICAL COMPOSITION OF WILD SOYBEAN SEED COAT RELATED TO ITS PERMEABILITY

DANG TOAN VU[2], VIJAYANAND VELUSAMY[1,3] AND EUIHO PARK[1*]

[1]*School of Biotechnology, Yeungnam University, Daegu, Korea*
[2]*Research Planning and International Cooperation Division, Plant Resources Center, An Khanh, Hoai Duc, Ha Noi, Vietnam*
[3]*Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, 1266 Sinjeong, Jeongeup, Jeonbuk, 580-185, Korea*
**Corresponding author's e-mail: ehpark1@ynu.ac.kr ; Ph: 82-53-810-3022; Fax: 82-53-810-4769*

Abstract

Four wild and two cultivated soybeans were examined for the water imbibition and relationships with seed coat structure, histochemical and epicatechin content using light, fluorescence, electron microscope, and HPLC technique. Cultivated cultivars, Pungsan and Chungja showed the highest water permeability, while the wild soybean lines, YWS16 and YWS136 showed moderate, and YWS1420 and YWS1459 did not absorb water for 24 hours. Seed coat surface of Pungsan, Chungja, YWS16, and YWS16 showed free deposits with rarely thin deposits exposing a cutin layer, while those of YWS1420 and YWS1459 were thick, rarely exposing the cuticle. In permeable genotypes, several cracks were found and the size of the cracks ranged from 1 to 2 µm. However, in impermeable genotypes, the cracks were not found in all seeds except for the tiny cracks (0.1 - 0.2 µm) in YWS1459. The free deposit with lager cuticle cracks on the seed coat surface seems to be related to permeability of wild soybean seed coat.

Key words: Wild soybean, Seed coat, Epicatechin, SEM and Water imbibition.

Introduction

Seed coat plays an important role in the process of water imbibition and prevents seeds from soaking damage or soaking injury during seed germination or food processing (Ma *et al.,* 2004; Qutob *et al.,* 2007; Shao *et al.,* 2007; Koizumi *et al.,* 2008). Further, seed coat protects seeds against deterioration and makes seeds maintain high quality and viability through lengthy storage (Zhou *et al.,* 2010). The soaking damage of seed takes place in the early stage of imbibition (Parrish & Leopold, 1977; Meyer *et al.,* 2007). The tissue hydration occurs in a controlled way and the internal structures of the cell and organelles are not affected due the presence of seed coat (Meyer *et al.,* 2007). Seed impermeability or permeability is determined by the seed coat (Shao *et al.,* 2007). Water uptake is an important step towards the initiation of biochemical changes that lead to germination completion (Zamin *et al.,* 2010).

Strong resistance to water is considered as hard seededness. Such seeds do not readily imbibe water when soaked, even for prolonged periods and under favorable conditions (Rolston, 1978; Ma *et al.,* 2004). Hard seededness is considered as coat-imposed dormancy, or physical dormancy.

The barrier system to water permeability in the hard seeds occurs in two ways, structure and chemical composition. A complex multilayer seed coat can function as a barrier to water. The other is due to the presence of chemicals in a continuous layer of cells in the seed coat. The presence of phenolic compounds is particularly important for seed coat impermeability (Chachalis & Smith, 2001; Zhou *et al.,* 2010). Epicatechin is a phenolic compound, which is positively correlated with the change of hard seed percentage under different water and gas conditions (Zhou *et al.,* 2010).

So far, however, there has been little information about water imbibitions of wild soybean seed coat, anatomical structure, histochemistry, and epicatechin content of seed coats. The main issues studied in this paper are: a) differences in seed coat anatomy related to permeability properties of wild and cultivated soybeans, b) location for water entering and moving into the seed, c) relationship between seed coat epicatechin content and permeability of seed coat.

Materials and Methods

Plant materials: Six genotypes including two permeable wild soybean lines (YWS16 and YWS136), two impermeable wild soybean lines (YWS1420 and YWS1459) and two permeable cultivated soybeans Pungsannamulkong (Pungsan) and Chungjakong (Chungja) were used to test the water permeability of seed coat.

Measurement of uptake absorption: For each genotype, 1,000 mg of seeds were tested for water absorption. Imbibed seeds were weighed every hour for 12 hours and the final measurement was taken after 24 hours. Individual seed weighed before immersing in tap water. Each seed soaked in water, placed on paper towels to remove surface water, and weighed. The rate of water absorption was standardized by expressing as weight increase (mg) per original seed weight. Seeds without imbibition for 24 hours were classified as impermeable.

Scanning electron microscope (SEM): Seed coat surfaces were observed under SEM. The abaxial seed side containing hilum, micropyle and raphe, and the dorsal side were sectioned into 2 mm^2 transverse segments, and immediately placed into vials of FAA fixation [3.7% (v/v) formaldehyde, 50% (v/v) ethanol, and 5% (v/v) acetic acid] for 24 h at room temperature. After fixation, the sections were rinsed three times in 50% EtOH, and dehydrated in a series of ethanol solutions, 70%, 90% and 95% for 10 min. The sections were stored in absolute ethanol for 1 hour, and then coated with gold and examined with a Hitachi S570 scanning electron microscope. Five seeds were examined for each genotype.

Sample preparation for anatomy and histochemical analyses: The seed coats were fixed and sectioned into specimens according to Pandeya *et al.,* (2007). The abaxial seed side containing hilum, micropyle and raphe, and the dorsal side were sectioned, and they were fixed in FAA fixative mix solution containing 50% of 95% EtOH, 5% of glacial acetic acid; 10% of 37% formaldehyde and 35% ddH_2O overnight at 4°C. On the next day, the samples were rinsed with 50% EtOH and dehydrated using 60%, 70%, 80%, 85%, 90% and 95% EtOH for 10 min each, and then incubated overnight in 95% EtOH, and once again incubated for 30 min in 100% EtOH. They were cleared in 25% Xylene: 75% EtOH, 50% Xylene: 50% EtOH and 75% Xylene: 25% EtOH for 30 minutes each. The samples were incubated twice in 100% Xylene for 30 min each. The samples were slowly infiltrated in paraffin wax. Twenty chips of paraplast were put into a vial containing 1/2 Xylene and incubated at 42°C for 30 minutes. The paraplast in sample sections were completely dissolved in Xylene. This step was repeated until the vial was full of paraplast. Xylene paraplast solution was poured off; added molten paraplast and incubated at 60°C for 4 hours. This step was repeated three times. The samples were arranged in a hot boat and moulded with paraplast. The paraffin boat were then stored in 4°C for paraffin de-polymerization for 12 hours. The paraffin block were cut using a rotary microtome (MICROM GmbH, D-6900 HEIDELBERG, GERMANY) into 5-8 µm sections with Feather Blades. The cutting ribbons were floated on 42°C water bath for 5 minutes and took the ribbon on the slide. The slides were incubated on a slide warmer at 45°C overnight. To remove paraplast from the slides, the slides were dipped into the two changes of 100% Xylene for 30 minutes, 50% Xylene: 50% EtOH for 10 minutes, and 100% EtOH for five minutes. The samples were rehydrated by dipping through 95%, 80%, 70%, 50%, 30%, and finally a change of ddH_2O. The samples were used for anatomy and histochemical analyses.

Detection of the polycarboxylic acids and phenolic compounds: The detection of polycarboxylic acids and phenolic compounds was adopted by the method of O'Brien *et al*., (1964). Dehydrated tissues were stained with 0.05% toluidine blue. Toluidine blue O was dissolved in 100mM Na phosphate buffer of pH 3 and filtered with Whatman's filter paper. This solution was dropped on the slide with specimens; the samples were stained for five minutes and the excess stain was removed by dipping the slides into two changes of dH_2O for three minutes. The tissues were covered with a cover glass and observed under a light microscope (Olympus BX51 microscope). Images were recorded by an INFINITY camera (Numeria, Canada).

Detection of cutin: The method used for the detection of cuticle was followed by the method of Ma *et al*., (2004). Seeds were immersed in 0.001% aniline blue for 2 hours, rinsed in dH_2O three times, in xylene twice and observed under a fluorescence microscope with blue light (filter set: exciter filter BP 546, dichroitic mirror FT 580 and barrier filter LP 590) (Olympus BX51 microscope). Seeds were immersed in water until they were fully hydrated. A small wound was made at one or both ends of the impermeable seed coats to initiate hydration. Sections were stained for cuticle aniline blue. To obtain an overall view, seed coat tissues were observed under a fluorescence microscope with blue light (filter set: exciter filter BP 546, dichroitic mirror FT 580 and barrier filter LP 590) and recorded by an INFINITY camera (Numeria, Canada).

Detection of lignin: Detection of lignin followed the method of Ruzin (1994). Hydrated seed coat samples were stained with 0.01% Safralin for 24 hours, and rinsed with distilled water three times. Samples were counter-stained with fast green FCF for 10 min and washed with distill water three times, dehydrated in a series of EtOH and then observed with a light microscope. Lignin was shown as gray red.

Detection of neutral sugar: Sections were stained with periodic acid schiff (PAS). PAS was prepared by dissolving 1 g of basic fuchsin (Sigma-Aldrich) in 200 mL of boiling dH2O, cooled the solution to 70^{o}C and added 2 g of sodium metabisulphite, cooled to room temperature, added 10 mL of 1 N HCl, left for 24 h in the dark, added 2 g of activated charcoal, mixed well, and then filtered in solution. De-waxes sections were incubated in 1% periodic acid for 10 min, then rinsed in distilledwater three times, covered with Schiff's reagent for 10 min, washed in running tap water, counter-stained in Harris' hematoxylin, rinsed in distilled water, dehydrated through a series of ethanol concentrations (70%, 80%, 90%, 100%), cleared in xylene, and mounted, then observed under a light microscope.

Epicatechin content analysis: Same soybean genotypes used in the water uptake experiment were used. The standard chemicals of (-) epicatechin (EC, purity 99.7%) were purchased from Sigma Chemical Co. (Korea). Solvents and water including spectroscopy-grade acetonitrile, ethyl acetate and methanol, were purchased from Sigma.

Stock solutions of epicatechin 500 µg/mL were prepared by dissolving referent standards into the mobile phase. Less concentrated solutions including: 20 µg/mL, 50 µg/mL, 100 µg/mL, 150 µg/mL and 200 µg/mL, were prepared, as needed for standard for mobile phase, by diluting in the same mobile phase.

The seed coats were dissected from seeds and frozen in liquid nitrogen. Freeze-dried seed coats were ground to a fine powder. The fine powders (0.1 g) were extracted three times with 3 mL of 1% HCl/ 60% CH_3OH in dry ice. The combined extracts were made up to 10 mL. After centrifuging and filtering through a 0.45 µm membrane filter, 20 µL sample was injected into the HPLC column. The temperature for extraction of the samples was set at 4ºC.

The HPLC was conducted using the Yoonglin Chromatographic system – Model YN 9120 (Yoonglin, Korea) – a sample injector with a 20 µL sample loop. An Autochro-300 software was used for both the operation of the detector and for data processing. The conditions of HPLC were as previously described (Zhou *et al.,* 2010).

Temperature and humidity treatment: For the effect of humidity, the humidity chamber was set at 100% and 40°C. Seeds of each genotype were kept in the cylinders, then placed in the vials, and filled with 50 ml distilled water. Vials were kept empty in the humidity chamber for a day to keep stable humidity. Seeds were laid into those cylinders. Cylinders were placed in big vials and coved with polyvinyl.

Results

Water imbibition properties of six soybean genotypes: Water absorption of six genotypes are shown in Fig. 1. Two cultivated soybean varieties took up water faster than permeable wild soybeans(YWS16 and YWS136). Total water apsorption was higher in Chungja and Pungsan varieties than other wild soybean genotypes, and weight of water uptake of Pungsan and Chungja were increased 2.4 and 2.5 fold, respectively. Absorbed weight of permeable seeds of wild soybean lines, YWS16 and YWS136, increased 2.1 and 2 fold of dry seeds, respectively. The impermeable lines, YWS1420 and YWS1459, did not take up water for 12 hours. Similar results were reported by Ma *et al.,* (2004), in which permeable genotypes absorped from 2.2 to 2.5 fold of its original seed weight. Within 1 hour, water absorption of cultivated soybeans was faster than wild soybeans: Chungja and Pungsan, absorbed 416 mg and 383 mg, while YWS16 and YWS136 did 197 mg and 297 mg per 1,000 mg seeds, respectively. After 12 hours in tap water, Chungja and Pungsan showed higher water absorption (1,534 mg and 1,497 mg per 1,000 mg seed, respectively) than wild soybeans YWS16 and YWS136 (1,174 mg and 1,242 mg per 1,000 mg seed, respectively). The water imbibition of three genotypes, Chungja, YWS16 and YWS136, showed a linear pattern, while Pungsan showed a slightly curved pattern.

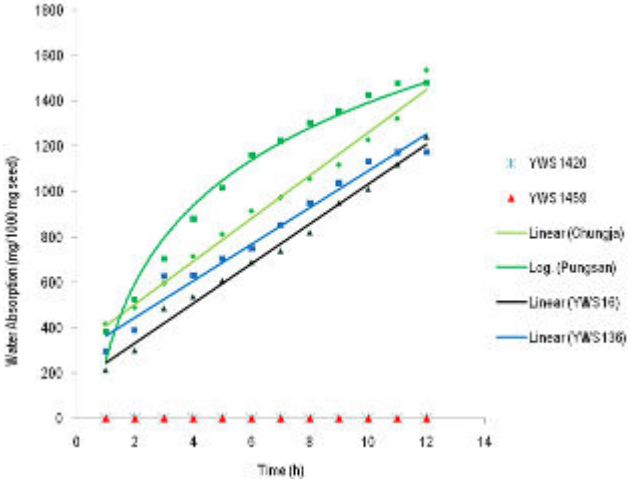

Fig. 1. Water uptake patterns of two cultivated soybean and four wild soybean genotypes with different permeable properties.

Initial site for water entering into seed coat: Initial site for water entry into the seed is shown in Fig. 2. Some wrinkles appeared on the dorsal side, and these wrinkles were initially found in cultivated soybeans after submerging into distilled water for 15 minutes. However, in permeable wild soybean lines, wrinkles appeared within 120 minutes after incubation and extended toward the ventral side (Fig. 2A, B and D). No wrinkles were found on YWS1420 (Fig. 2C). Fluorescence trace dye was used to detect initial water entry into the seed coat. If such sites were large enough to admit fluorescent, tracer dye molecules, the permeable areas could be visualized by a fluorescence microscope. Seeds were incubated in 0.001% coriphosphine O or aniline blue dye, and cultivated soybean seeds were incubated for 1 hour, and 5 hours for 2 wild permeable seed lines. Results were shown in bright wall staining of single or clusters of palisade cells with expanded Lumina (Fig. 3A, B and C). With very permeable seeds, staining was rarely observed in the periclinal walls of the palisade layer even after a short treatment time (Fig. 3C). Points of staining were on the dorsal side of the seeds. Ma *et al.,* (2004) reported similar finding in which initial sites of water entry into permeable seeds were on the dorsal side of seeds with some winkles. Later, tracer dyes expanded on larger areas. Ridges of dye were enlarged until abaxial and later on hilum (Fig. 3C).

Anatomy and histo-chemical analysis of seed coat

Features of dorsal side of seed: Seed coat structures of YWS1420 and YWS1459 were observed under scanning electron microscope (SEM) and shown in Fig. 4. The seed surface was scrobiculate or honeycomb in both YWS1420 and YWS1459. Seed coat at the dorsal side of YWS1420 and YWS1459 was heavily covered with deposited materials. They presented as a compartmentalized pattern, and the deposits were observed as materials attached to the surface of the seed coat. Deposits on the surface of YWS1420 seeds were embedded in different densities (Fig. 4A). These deposits are called seed blooms (Qutob *et al.,* 2007) or reticulum. The deposits were connected together, and they were laid on the surface of the cuticle palisade layer of the seed coat (Fig. 4B).

Furthermore, deposits enveloped an extensive area of the seed coat were found in YWS1459. They were laid out on the seed coat surface, but not connected to each other in some areas. Free seed coat deposits were found at some areas of the seed coat in YWS1459 (Fig. 4C and D). Cutin layer was clearly exposed in some areas where the deposits were not covered. This cuticle was rugulose. Most of YWS1459 seeds had no cracks, but rare cracks were found at the deposit areas of YWS1459. The cracks were tightly crack from 0.1 to 0.2 µm (Fig. 4D). Those deposits were quite different from each other. Ma *et al.,* (2004) also reported different types deposits that included light deposits, moderate deposits and heavy deposits. In addition, Qutob *et al.,* (2007) found deposits that appeared either randomly distributed or in a honeycomb-like pattern. The deposits were presumably waxy and lipid.

Seed coats of YWS16 were rarely distributed on the surface so that the cuticle was exposed (Fig. 5A). Similarly, YWS136 showed very few surface deposits. The deposit layer was covered with limited accumulation of crystal-like substances. Additional deposits covered several areas of the YWS136 seed coat, and cuticle was not extensively exposed (Fig. 5C). Fragments of endocarp tissues were observed where endocarp cells were sometimes discernable (Fig. 5C). Some cracks were found in the seed coat surface of WYS16 and YWS136. The size of these cracks were about 1-2 µm (Fig. 5).

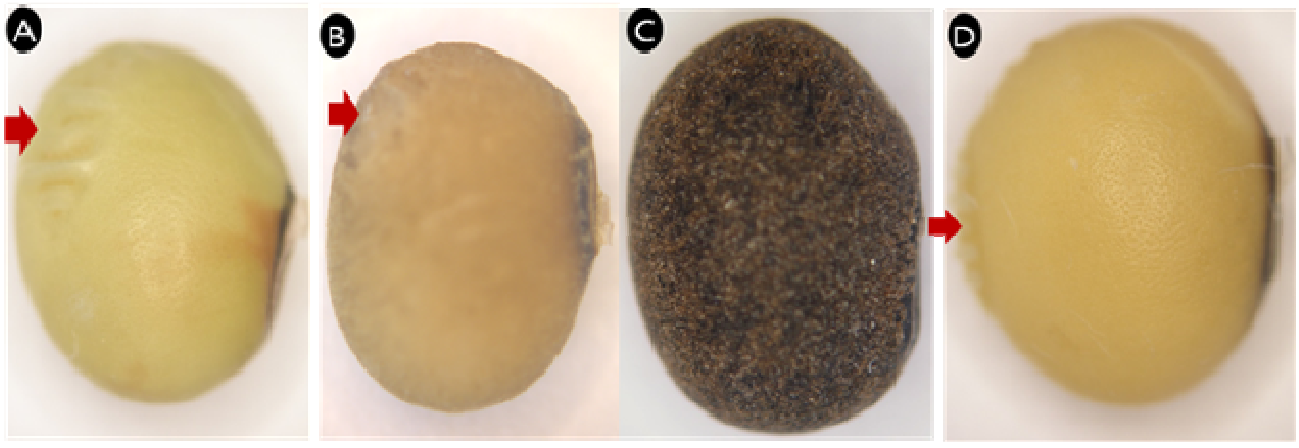

Fig. 2. Seed coat appearance after 2 hours of soaking in distilled water *[(A) permeable YWS16, (B) permeable YWS136, (C) impermeable YWS1420, and permeable YWS136. (Red arrows indicate the initial sites of water entry*)].

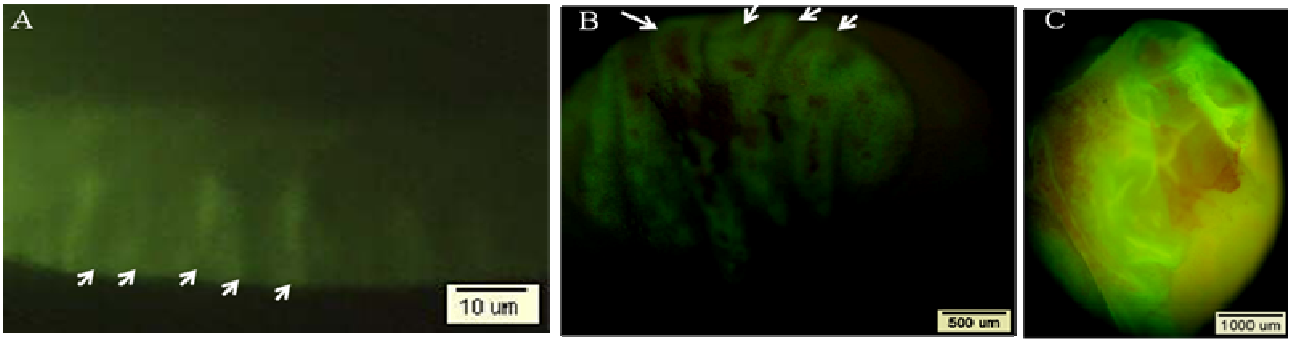

Fig. 3. Seed coat appearance after incubation in coriphosphine O *[(A) Permeable periclinal walls of the palisade layer in YWS136 after 2 hours, (B) Dye movement after 2 hours in the seed coat of YWS136, (C) dye movement after 5 hrs in YWS16 (Arrows indicate the initial sites of dye invasion)]*.

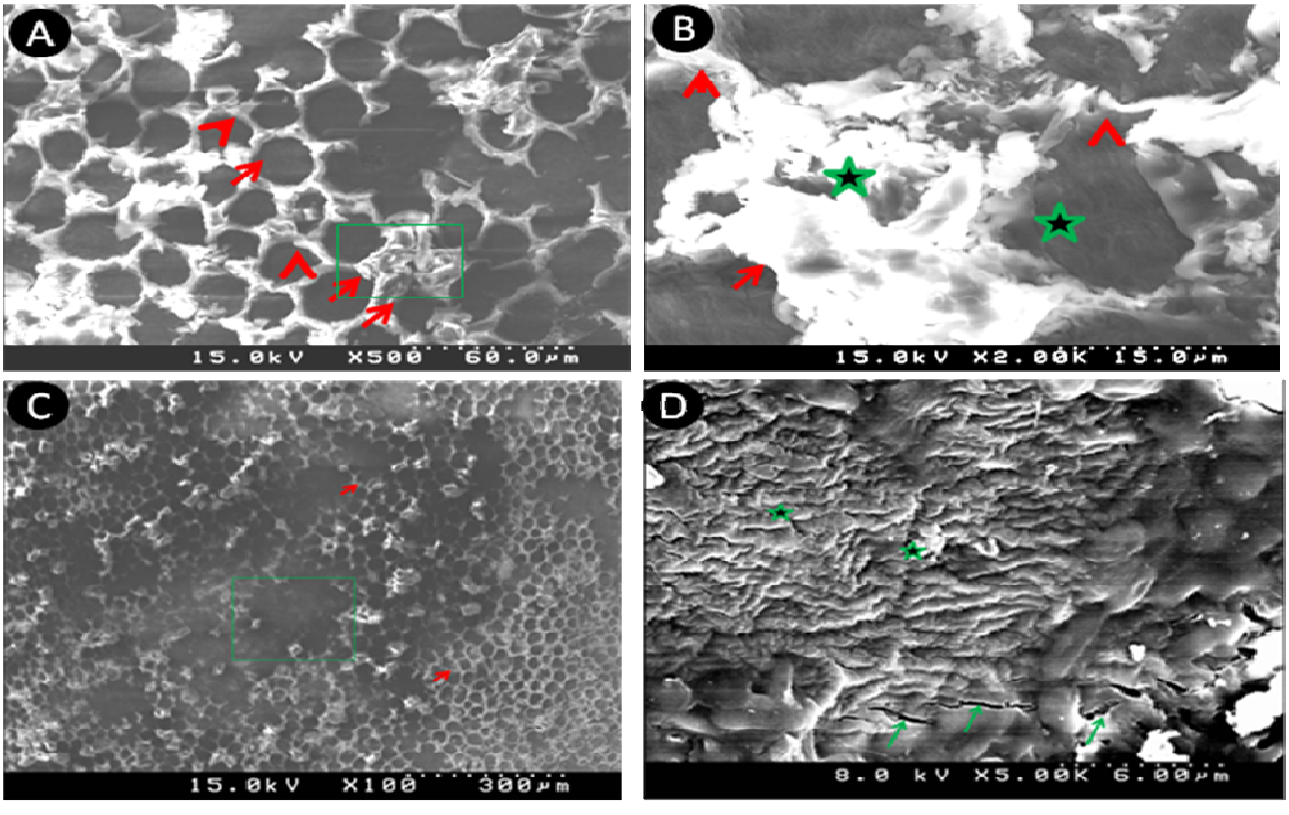

Fig. 4. Seed coat surface features of YWS1420 *[(A) heavy deposits, (B) higher magnification of (A)* and YWS1459 *[(C) heavy endocarp deposits found in YWS1459, (D) higher magnification of (C)]* on the dorsal side observed under SEM *(stars indicate cuticle palisade layer, arrows indicate the deposits, arrow heads indicate the deposit connections).*

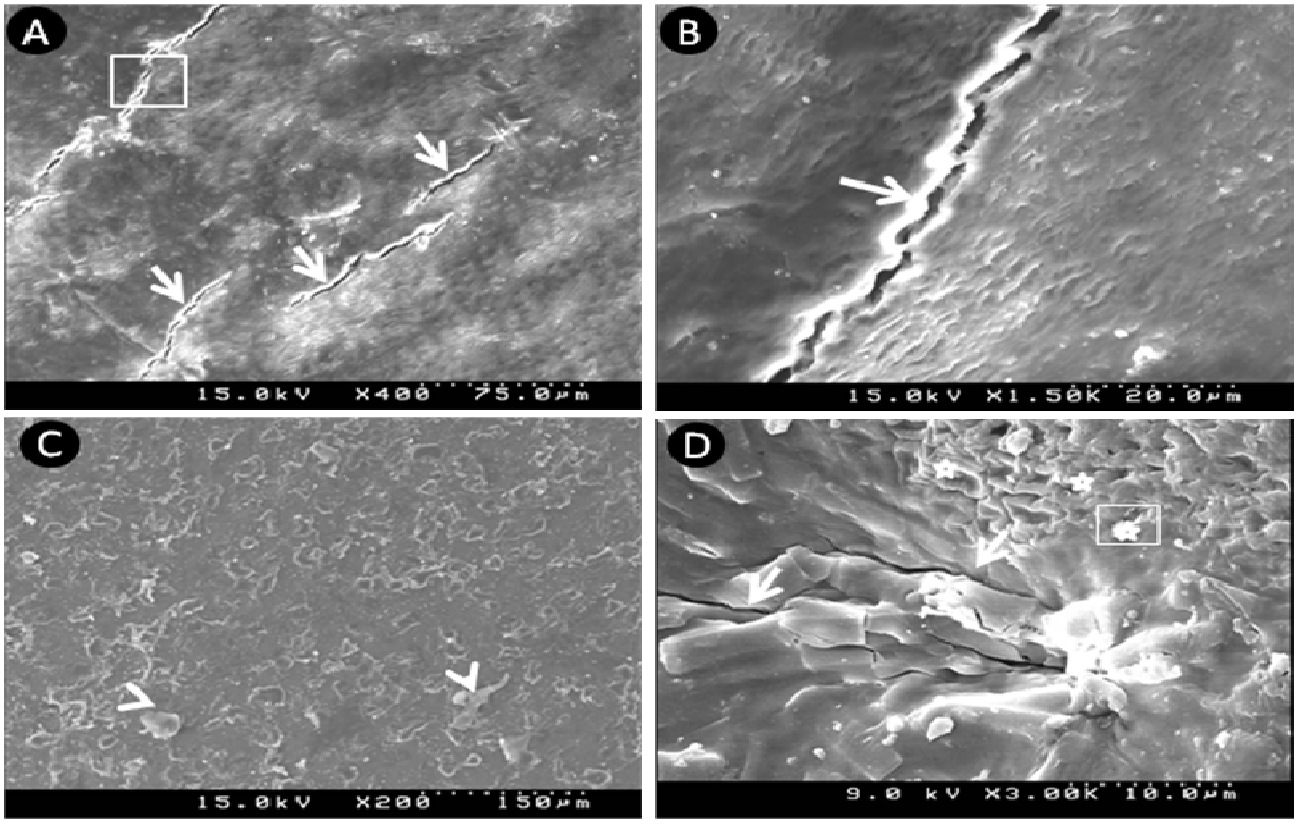

Fig. 5. Surface structures on the dorsal sides of YWS16 and YWS136 under SEM *[(A) YWS16 with several cracks in the surface area and free deposits (arrows showing the cracks); (B) YWS16 at higher magnification of (A) with a large crack (arrows indicate the surface crack). (C) YWS136 showing thin deposits (arrowheads indicate deposits). (D) YWS136 at depression with several cracks (stars indicate cuticle layer and arrows indicate cracks)]*.

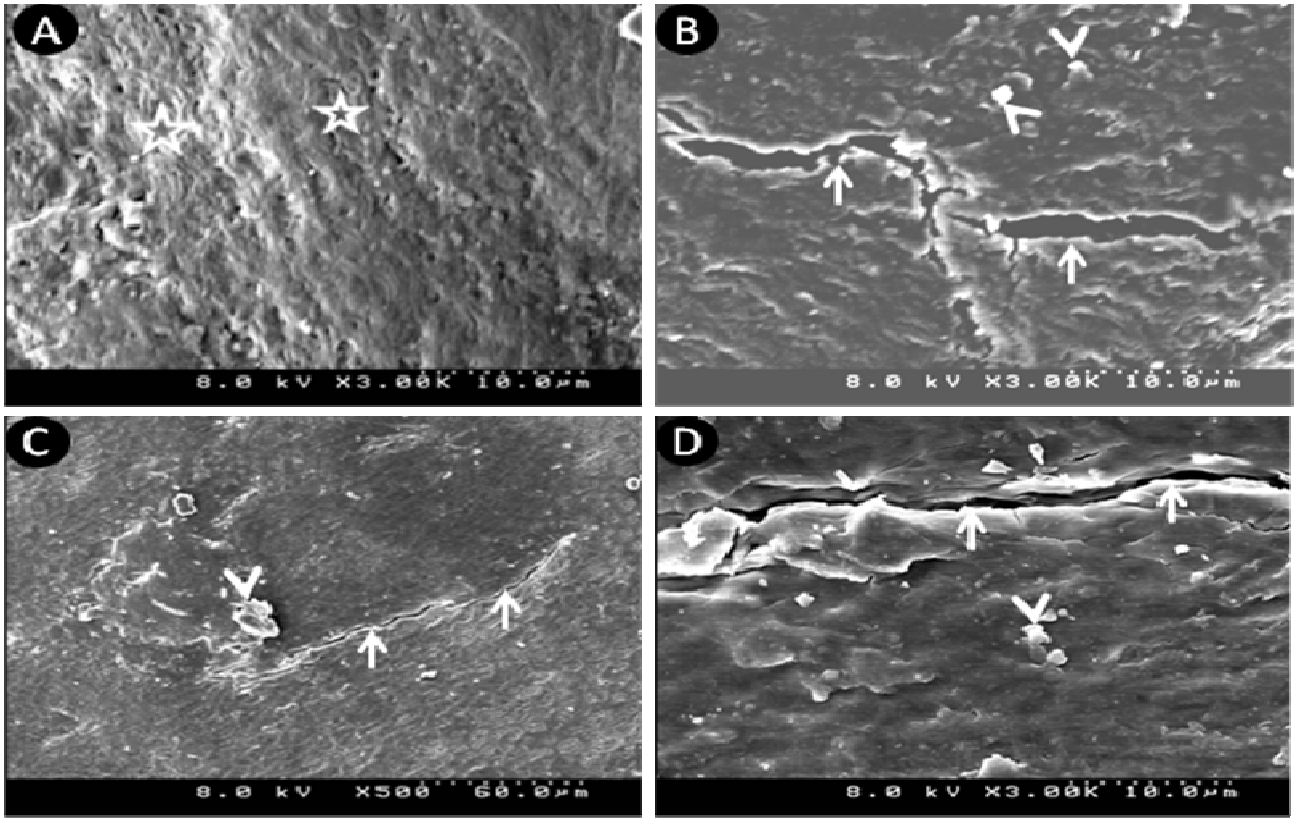

Fig. 6. Surface structure on the dorsal side of Pungsan and Chungja under SEM *[(A) Pungsan showing some pits and rough surfaces (green star indicates the rugulose cuticle). (B) Surface area of Pungsan at high magnification (green arrows indicate cutin cracks, red arrows indicate surface deposits); (C) Chungja (red arrows indicate deposits, green arrows indicate cuticle cracks). (D) Chungja seed coat surface at higher magnification (Green stars indicate the seed coat deposits and red arrows indicate cracks at the dossal site)]*.

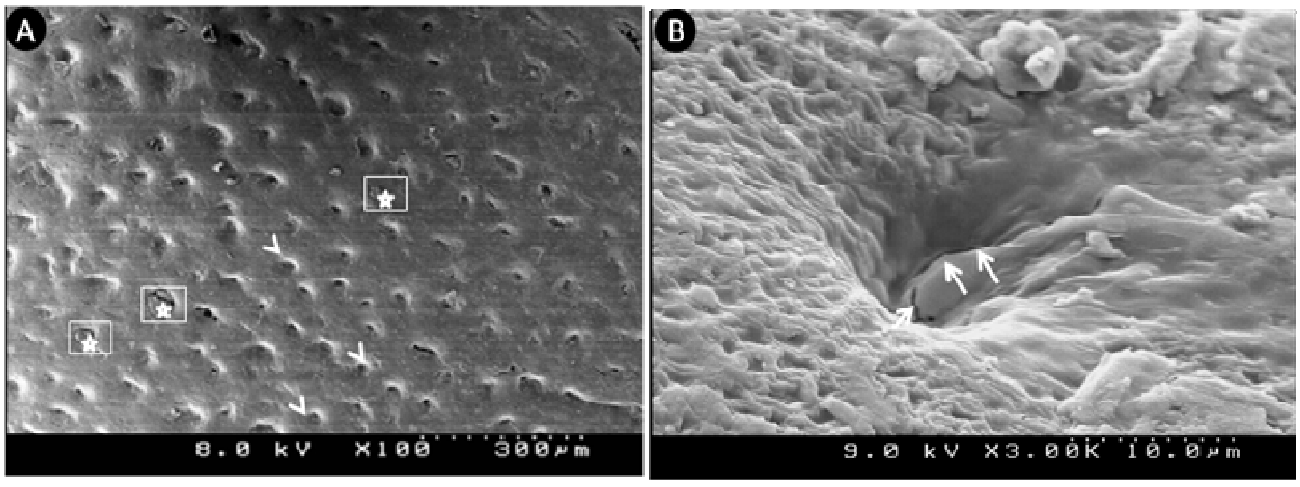

Fig. 7. Pores (depressions) on the dorsal sides of seed coat in Pungsan and YWS136 by SEM *[(A) Pungsan showing many pores with variable shapes (arrowheads indicate pits, stars indicate the deposit on the pits), (B) larger pit of YWS136 on the dorsal side with higher magnification (arrows indicate small cracks inside of the pit)]*.

Most dorsal areas of Pungsan were free of surface deposits (Fig. 6A) and amorphous deposits were rarely placed in the upper areas of the seed coat (Fig. 6B). Several cracks appeared in the cuticle layer of the seed coat (Fig. 6A and B). A rugulose shape of the cutin layer was found in Pungsan. Surface areas of Chungja showed thin deposits placed in the upper cuticle layer; some large cracks were also found in the cuticle layer of Chungja with the sizes around 1-2 µm (Fig. 6C and D). The cuticle layer was determined to be smooth. Surface deposits found on the seed coat surfaces of Chungja and Pungsan were different from wild soybeans (Fig. 6A, B, C and D).

Many cracks were found in soybean seed coat under SEM. These cracks were narrow splits (Fig. 4B, 6B and 7B) and were frequently found on the dorsal side of seeds, however, no cracks were found on the abaxial side of seed coat. Cracks varied in size ranging from 0.1-0.2 µm in YWS1459 (Fig. 4B) and YWS136, while Pungsan, ones of Chungja were 1-2 µm (Fig. 6B). Cracks also varied in depth. It was very difficult to measure the depth of the cracks. The cracks typically occurred through the outer periclinal cells as well as the cuticle or even cell layers underneath (Fig. 5B and D; 6B and D). The cracks observed under tracer dye are shown at Fig. 3A. These figures suggested that the permeability of soybeans in the cultivars were related to cracks. Even though cracks were found in permeable YWS16, YWS136, Chungja and Pungsan, cracks may not be the main reason for the permeability of wild soybeans.

Several pits were observed in Pungsan and YWS136 seed coats under SEM. Pits were normally found on the dorsal side (Fig. 7A). On the abaxial side, pits were not found. Many pits were found in Pungsan (Fig. 7A), and this was different from the results of Ma *et al.,* (2004) where more pits were found near the hilum. There were three types of pits (pores) observed in the soybean seed, namely: shallow-elongated, deep circular, and filled with deposits (Fig. 7A). Several cracks were found beside the pits of the YWS136 genotype when those pits were observed under higher magnification. Previous reports found three kinds of pits, namely: deep-elongated, deep-circular, and shallow circular (Chachalis & Smith 2001). Diverse pits in both shape and shallow were found, however, pits were not found in Chungja, YWS16, YWS1420 and YWS1459, since pits were only found in permeable genotypes (Pungsan and YWS136). Two impermeable seed genotypes as well as two permeable Chungja and YWS16 were not shown to have any pits.

The features at abaxial site of seed: The abaxial structure of soybean seeds was extensively studied using SEM. YWS16, Pungnan, YWS1459, and Chungja seeds had a large cleft in the hilum that was formed by cell separation during seed maturation (Fig. 8A, C, E and F), while YWS136 and Pungsan did not have any narrow cracks in the hilum (8B and D). The hilum of YWS16, YWS1420 and Pungsan were opened (Fig. 8A, C and D), whereas YWS136, YWS1459 and Chungja varieties were closed. Micropyle of matured YWS16, YWS136, YWS1420, YWS1459, Pungsan and Chungja seeds were closed. Raphe of YWS136 and Pungsan seeds were opened, whereas the ones of YWS16, YWS1420, YWS1459 and Chungja were closed. Several deposits were found on the abaxial side of the seeds, whereas some areas showed clear cuticles. No cutin crack was found in other areas.

Palisade layer: Palisade layer was developed from the outer epidermis of the outer integument (Ma *et al*., 2004), which does not have any intercellular space, with uneven construction of the cell wall. There was only a primary wall at the outer periclinal end; the anticlinal wall was unequally thickened in addition to thickening along the entire length (Fig. 9A and B). In a hydrated seed coat, palisade cells were swollen so that cell lumina expanded and some outer tangential walls were broken (Fig. 9B and 9A and C). Whereas, the palisades of YWS16, YWS136, Chungja and Pungsan showed light red (Fig. 9A and B), YWS1420 and YWS1450 were faintly stained with PAS showing grey-red (Fig. 9C).

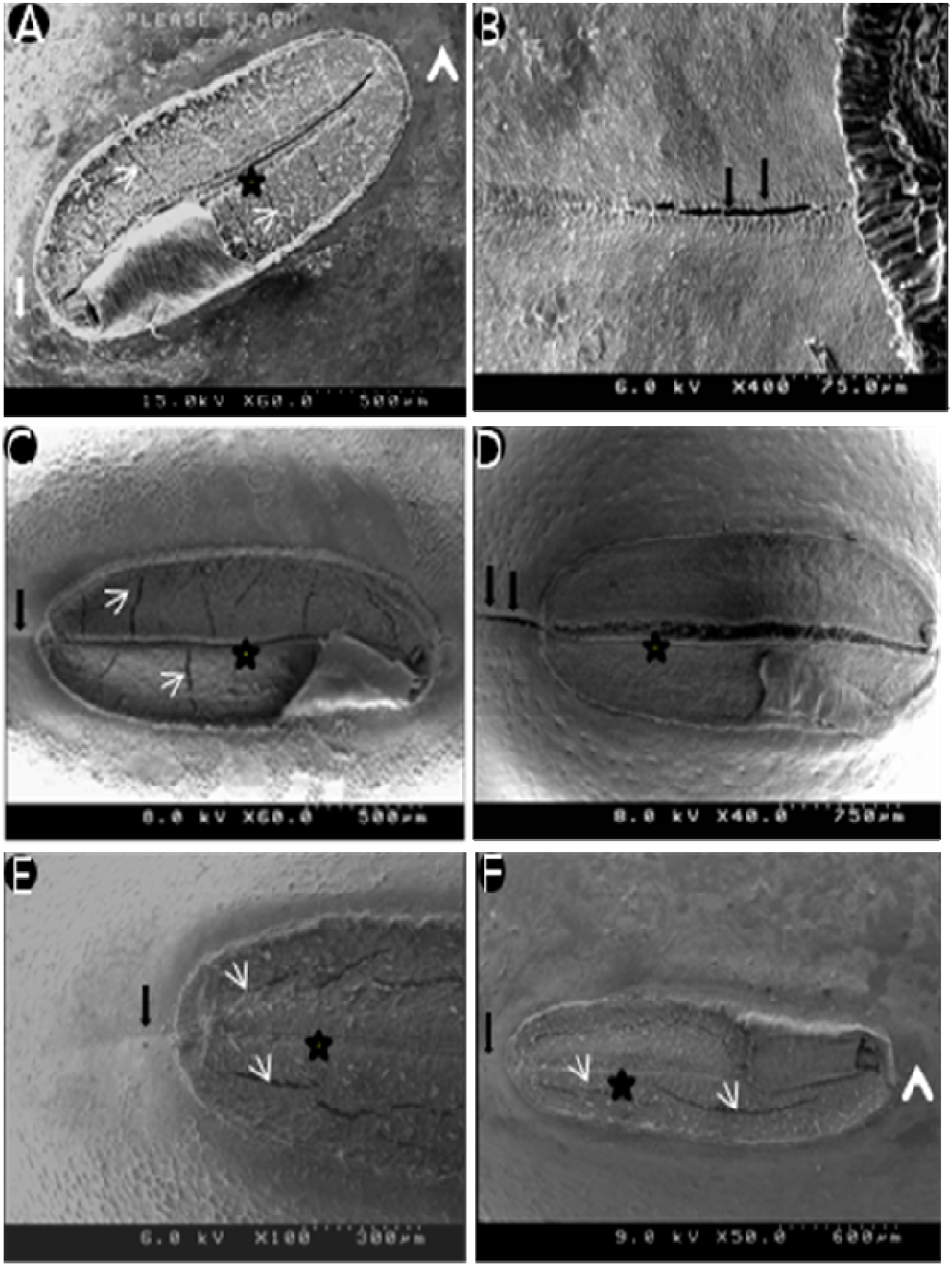

Fig. 8. Abaxial side of cultivated and wild soybean seeds under SEM *[(A) cracks (arrows), opened hilum (star) and closed raphe (arrowheads) of YWS16. (B) opening raphe (black arrows) of YWS136. (C) cracks in hilum, opened hilum (star), and closed raphe (black arrow) of YWS1420. (D) no crack in hilum, opening hilum (star), closed raphe (black arrows) and closed micropyle (arrows) of Pungsan. (E) cracks in hilum (arrows), closed hilum (star), and close raphe (black arrows) of YWS1459. (F) cracks in hilum, closed hilum (star) and closed raphe (black arrows) of Chungja]*.

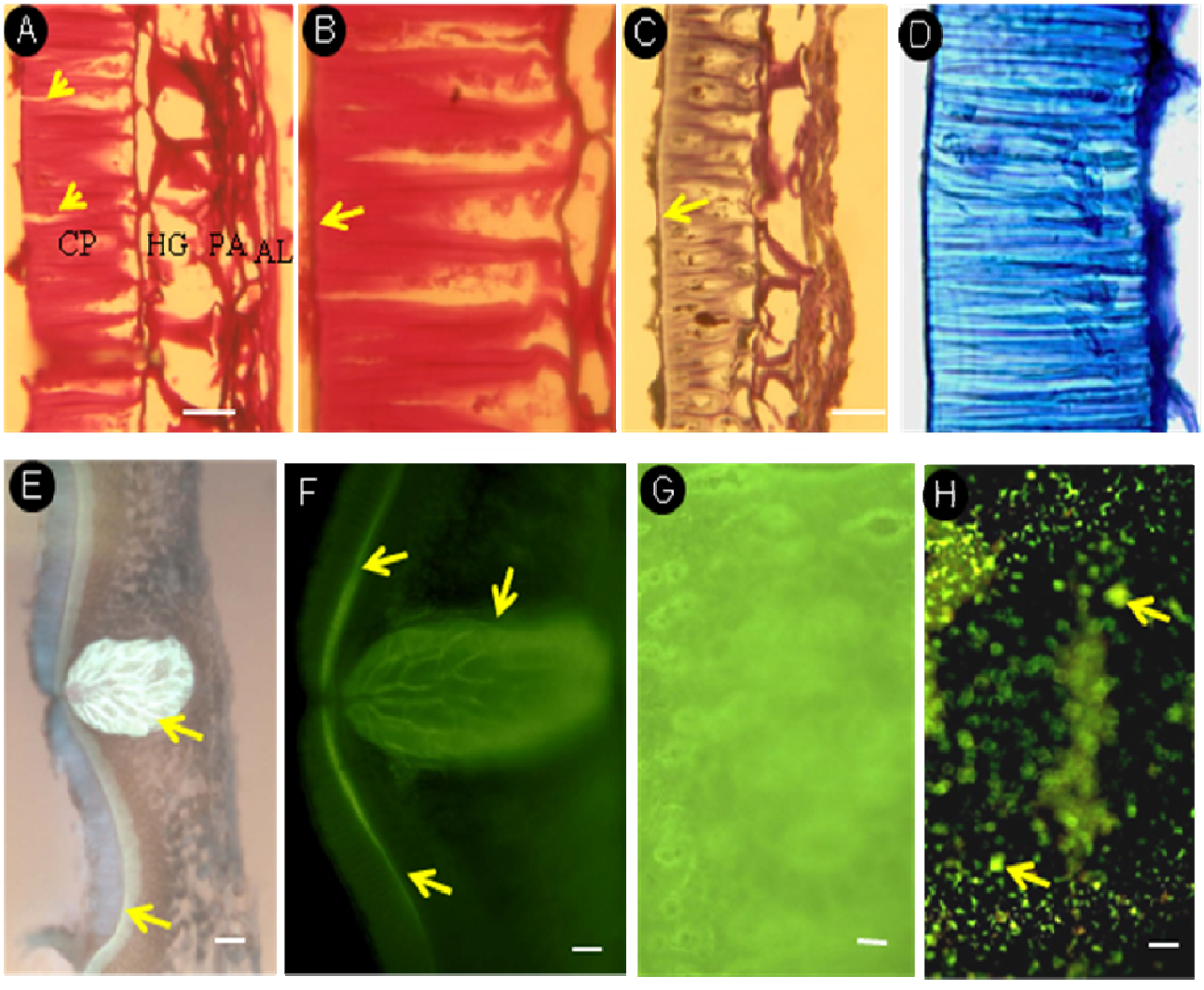

Fig. 9. Structure and histochemistry of seed coat *{(A) Seed coat section of Pungsan on the dorsal was stained with periodic acid schiff (PAS) and observed under light microscope. [counterpalisade layer (CP), hourglass layer (HG), compressed parenchyma (PA), aleurone layer (AL)]. (B) Enlarger of (A) showing intracellular space at palisade. (C) Cross section on the dorsal site of YWS1420 showed grey-red (arrow indicates light line). (D) Palisade of Pungsan stained with aniline blue. (E) Hilum of Pungsan stained with phloroglucinol-HCl [counterpalisade layer (CP) showing violet], tracheid bar (TB)]. (F) Hilum site of Pungsan stained with analine blue. (G) Seed coat surface of YWS16 stained with analine blue (H) Seed coat surface of YWS1420}.*

Surface deposits did not stain with analine blue (fluorescence dye), thus the seed coat had a dark appearance. While the cuticle was highly exposed with a green-yellow color under UV light, some cracks were found in the hilum region of YWS16, YWS1420, YWS1459 and Chunja. However, cracks were not found at the hilum region of YWS136 and Pungsan (Fig. 9D). Seed coat surfaces of YWS16, YWS136 and Pungsan were similarly exposed with UV light and showed yellowish green (Fig. 9G), however, YWS1420 and YWS1459 were not exposed with UV light and showed dark black, where the cuticle was not covered with deposits, and several yellowish green spots exposed the cuticle (Fig. 9H). Seed coat surface of YWS1420 and YWS1459 were covered with thick deposits in Fig. 4, and the cuticle was exposed in the seed coat surface of YWS16, YWS136, Pungsan, Chungja in Fig. 5 and 6.

Hourglass layer: This layer was made of osteosclereids (Ma *et al*., 2004), which is shown in Fig. 9A and C. Cells near the hilum were the longest which were arranged in the anticlinal direction and those in the dorsal region were short (Fig. 9A and C). No difference was detected among the genotypes regarding the hourglass layer.

Parenchyma and Endosperm: A few layers underneath the hourglass layer were severely compressed in a dry seed coat, but the cells could partially regain their original shapes when hydrated (Fig. 9A and C). All cells were connected to their neighbors by branches (Fig. 9A). There was no difference between wild and cultivated soybeans.

The aleurone was the outermost endosperm layer and was characterized by thick outer periclinal walls (Qutob *et al.,* 2007), and this showed a thin, and unevenly thickened anticlinal walls (Fig. 9A and C). No differences were detected among the cultivars regarding the endosperm.

Epicatechin content: Epicatechin was reported as a candidate factor affecting hard seed trait by Zhou *et al.,* (2010). Epicatechin contents in the seed coats of genotypes were presented in Fig. 11. Two permeable

YWS16 and YWS136 showed 0.54 µg/g and 0.68 µg/g, respectively, and were quite lower than impermeable YWS1420 (6.64 µg/g) and YWS1459 (8.47 µg/g). Content of cultivated cultivars Pungsan and Chungja were 0.007 µg/g and 24.56 µg/g, respectively. Epicatechin contents in the seed coat of hard seed lines, YWS1420 and YWS1459, stored under high humidity conditions are shown in Table 1. Results indicated that humidity treatment increased the content of epicatechin. The epicatechin content of YWS1420 and YWS1459 after treatment were 13.01 µg/g and 14.65 respectively, and in comparison with the control, they were increased by 49% and 57%, respectively.

Effect of high temperature and humidity treatment on hard seed: After treatment at the temperatures of 40°C, 50°C and 60°C, percentages of hard seeds in the YWS1420 genotype were 90%, 90% and 100 % respectively, while that of YWS1459 were 75%, 80% and 85%, respectively (Table 2). However, when hard seeds were imcubated in a 100% humidity chamber at 40^0C, seeds of YWS1420 and 1459 became permeable. Santana *et al.,* (2010) used six typical shrub or sub-shrub of *Fabaceae* family to break the hardseededness and concluded that high temperature (61^oC) could break hard seeds by 67% comapred to 21% at 28^oC for 10 days.

The changes in seed coat surface were observed under SEM, and results were shown in Fig. 10. Several larger cracks were found on the dorsal site of the seed. The deposits of humidity treated seeds were less prominent and thinner (Fig. 10) than the untreated seed (Fig. 4). This might occurred during incubation at high relative humidity where the deposits of seed coats were removed and the cracks exposed to cause permeablity.

Table 1. Epicatechin content (µg) before and after treatment with high humidity (mean ± standard deviation).

Accession No.	Control	Treatment
YWS1420	6.64 ± 0.42	13.01 ± 0.56
YWS1459	8.47 ± 0.77	14.65 ± 3.45

Table 2. Changes of hard seed ratio(%, hard seed) after high temperature treatment.

Accession No.	Temperature and humidity treatment				
	Control	At 40°C	At 50°C	At 60°C	100 % humidity
YWS1420	100	90	90	100	0
YWS1459	100	75	80	85	0

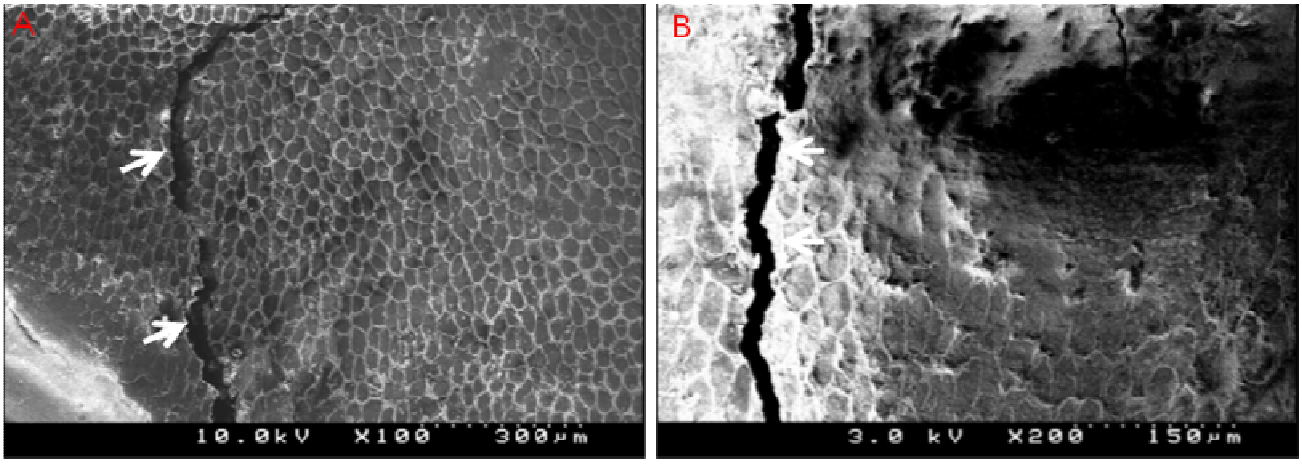

Fig. 10. Seed coat surface on the dorsal sites *[(A) YWS1420, (B) YWS1459 (arrows indicate the cracks after high humidity treatment)].*

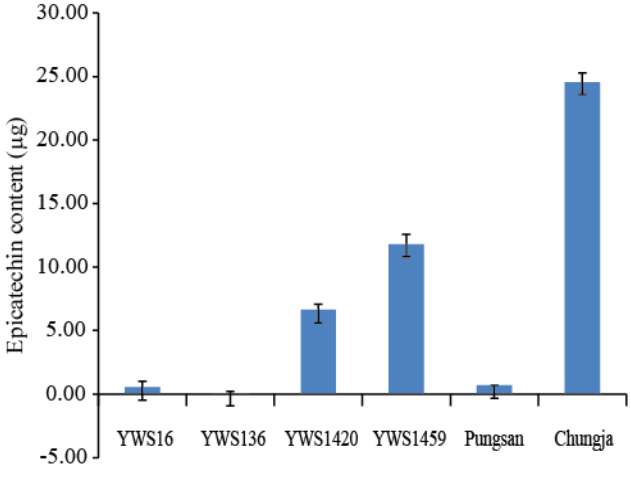

Fig. 11. Epicatechin content of seed coat in cultivated and wild soybean genotypes.

Discussion

Proper seed germination is vital for better crop yield (Munawar *et al.,* 2012). Seed coat plays an important role in the process of water imbibition (Ma *et al.,* 2004; Shao *et al.,* 2007; Meyer 2007; Koizumi *et al.,* 2008). The objective of this study was to give a whole picture of hard seed characters of wild soybeans and its relation to seed coat structure and chemical components. One hundred and ninety seven out of 200 wild soybean genotypes (98.5%) had hard seeds and only three wild soybean genotypes, YWS16, YWS67 and YWS136 showed permeable trait by pre-screening test. Hardseededness of wild species could help them survive in the soil for longer periods and can germinate at a favorable time. Hard seed was variable according to the genotypes and highly affected by the duration of temperature and water treatment.

Cell structure of the seed coat: Some legumes have intercellular space in the outer and inner hourglass layer except in the hilum region where they are absent (Jha & Pandey, 1989; De souza & Marcos-Filho, 2001). Cells of the hourglass layer on the dorsal side of this experiment were shorter than other regions (Fig. 9A and D). The aleurone layer is able to remove (McCleary & Matheson, 1976) and protect the embryo (Matsui *et al.,* 2005) during seed germination, however, it is not known whether it plays any role in the scheming of water imbibition. A cuticle layer is found on the aleurone layer. This cuticle is initially on the inner integument and, it does not control water imbibition because there are no differences among the permeable and impermeable cultivars (Chamberlin *et al.,* 1994; Ma *et al.,* 2004). Thus, this study did not focus on this cuticle layer. A previous report also explained that aleurone layers might involved in water uptake, however, they could not be a barrier for water because they were located deep in the seed coat. Therefore, they cannot affect the initial water uptake (Arechavaleta-Medina & Snyder, 1981; Ma *et al.,* 2004). As the structure of palisade layer and its surface features have been reported as a water barrier, detailed experiments focused on this layer.

Surface deposits and cuticle layer: This result might be the first report about amorphous deposits of the wild soybean seed coat. Impermeable wild soybeans, YWS1420 and YWS1459 were covered with surface deposits which were connected to each other and were placed on the upper surface cuticle. They distributed complicatedly and shapes of deposits varied within the seeds of each genotype. This feature was different from cultivated soybeans where a very thin layer of amorphous material and scattered upper cutin layer were observed (Ma *et al.,* 2004).

The outermost layer is a waxy cuticle that is considered as a first barrier to imbibition (De souza & Marcos-Filho, 2001). The deposits covering an extensive area of the seed coat are known as hydrophobic proteins in soybean. This protein is formed from the proclaiming super family and related to the structure of plant lipid transfer proteins. Hydrophobic proteins may control the adherence of the endocarp to the seed, but it structurally resembles the lipid transfer proteins (Gijzen *et al.,* 2006). The surface deposits may originate from the endocarp (Ma *et al.,* 2004). It is scratched of the fruit wall and stayed on the seed (Wolf *et al.,* 1981; Ma *et al.,* 2004). They constituted a hydrophobic layer comprised of a lipid and waxy coating on the surface of the seed coat (Ning *et al.,* 2005). In fact, deposits were prominent in impermeable YWS1420 and YWS1459 (Fig. 4) comparing less or no deposits in permeable YWS16, YWS136, Pungsan and Chungja (Fig. 5 and 6). Deposits of seed coat in permeable YWS1420 and YWS1459 were less prominent after treatment with high humidity and temperature (Fig. 10A and B). Wolf *et al.,* (1981) also concluded that permeability might be related with hydrophobic deposits, which was noticed by different responses of surface deposits to different solvents. The less permeable testa or high seed coat ratio may be the cause of slow water uptake (Aysun *et al.,* 2004).From these results and discussions, hydrophobic characteristic of deposits seemed to be related to water resistance in wild soybean species.

Cuticle was clearly exposed covering the palisade layer. Arechavalate-Medina & Snyder (1981) broke the impermeable soybean seeds to permeable ones by careful scraping off a small area of the outer layer of the seed coat (deposits and cuticle). Meyer *et al.,* (2007) found a small hole of permeable seeds after boiling impermeable seeds in NaOH. They concluded that the cuticle layer were broken, and the seed became permeable. In this experiment, some large cracks were found in four permeable genotypes. Seeds of YWS1420 and YWS1459 showed minute cuticle cracks thus they could not take up water. However, when the seed coat of impermeable hard seed was broken by high humidity and temperature treatment, lipid might be removed, therefore, the cuticle cracks were largely displayed.

Epicatechin and hardseededness: Epicatechin was positively correlated with the change of hard seed percentage (Zhou *et al.,* 2010). Hard seed ratio was increased at high temperature treatment (Table 2). In contrast, Epicatechin contents of Chungja, YWS1420 and YWS1459 were 24.56 µg/g, 6.64 µg/g and 8.47 µg/g, respectively, whereas Pungsan, YWS16 and YWS136 were 0.675 µg/g, 0.54 µg/g and 0.07 µg/g, respectively. Epicatechin was higher in the hard seed of YWS1420 and YWS1459 than permeable YWS16, YWS136 and Pungsan, but it was quite lower than the permeable genotype 'Chungja'. Its contents in addition to the hard seeds significantly decreased under high humidity and temperature conditions (Fig. 11). As the epicatechin content of impermeable YWS1420 (51%) and YWS1459 (57%) were comparatively higher than the untreated control, this result was quite different from Zhou *et al.,* (2010). Epicatechine was found to be combined with procyalidine to form an *ortho*-dephenolic compound, rarely found at mono-hydroxylated structures (Reichel *et al.,* 2011). Epicatechine of *ortho*-dephenolic form was not detected, however, in the process of the hard seed breaking under high temperature and humidity, the epicatechin might have converted to mono-phenolic and could be detected. Thus, the epicatechin content was quite higher than in the dry seed. Epicatechin contents in the seed coats of YWS1420, YWS1459 and Chungja with the black seed coat were higher than the ones of YWS16, YWS136, Pungsan with a yellow color. The epicatechin content might not be related to hard seed. Epicatechin content seemed to be involved to the seed coat color rather than hard seed.

Conclusions

The results of this experiment must be interpreted in the context of a number of potential limitations. The water imbibition and relationship with structure and chemical components were obtained from a limited sample with two permeable cultivated and two wild soybean and two impermeable wild soybean genotypes. However, our gene bank accessions only had three permeable wild soybean genotypes thus two permeable genotypes seemed reasonable in this study. The anatomy of seed coat might relate to water imbibition of seed coat in wild soybeans. In particular, the surface deposits and cuticle cracks seemed to be the major factors related to hardseededness.

Acknowledgement

This research was supported by the Yeungnam University research grants in 2011.

References

Arechavaleta-Medina, F. and H.E. Snyder. 1981. Water imbibition by normal and hard soybeans. *J. Am. Oil Chem. Society*., 58: 976-979.

Aysun, P., E. Peksen and H. Bozoglu. 2004. Relationships among some seed traits, laboratory germination and field emergence in cowpea (*Vigna unguiculata* (L.) Walp.) genotypes. *Pak. J. Bot*., 36(2): 311-320.

Chachalis, D. and M.L. Smith. 2001. Seed coat regulation of water uptake imbibition in soybean *Glycine max* (L) Merr.). *Seed Sci & Technol*., 29: 401-412.

Chamberlin, M.A., H.T. Horner and R.G. Palmer. 1994. Early endosperm, embryo, and ovule development in *Glycine max* (L.) Merr. *Int. J. Plant Sci*., 155: 421-436.

De souza, F.D. and J.M. Marcos-Filho. 2001. The seed coat as modulator of seed environment relationships in Fabaceae. *Revta Brasil. Bot., Sao Paulo.,* 24: 365-375.

Gijzen, M., K. Kuflu and P. Moy. 2006. Gene amplification of the Hps locus in *Glycine max*. *BMC Plant Biol*., 6: 6.

Jha, S.S. and A.K. Pandey. 1989. Seed coat structure in Melilotus (Fabaceae). *Phytomorphology,* 39: 221-229.

Koizumi, M., K. Kikuchi, S. Isobe, N. Ishida, S. Naito and H. Kano. 2008. Role of seed coat in imbibing soybean seeds observed by micro-magnetic resonance imaging. *Ann. Bot.,* 102: 343-352.

Ma, F., E. Cholewa, T. Mohamed, C.A. Peterson and M. Gijzen. 2004. Cracks in the palisade cuticle of soybean seed coats correlate with their permeability to water. *Ann. Bot.,* 94: 213-228.

Matsui, K., H. Hiratsu, T. Koyama, T. Tanaka and M. Ohme-Takagi. 2005. A chimeric AtMYB23 repressor induces hairy roots, elongation of leaves and stems, and inhibition of the deposition of mucilage on seed coats in *Arabidopsis*. *Plant Cell Physiol.*, 46: 147-155.

McCleary, B.V. and N.K. Matheson. 1976. Galactomannan utilization in germinating legume seeds. *Phytochemistry*, 15: 43-47.

Meyer, C.J., E. Steudle and C.A. Peterson. 2007. Patterns and kinetics of water uptake by soybean seeds. *J. Exp. Bot*., 58: 717-732.

Munawar, I., M. Dil, U.H. Zia, J. Yasir and M.R. Ahmad. 2012. Effect of pre-sowing magnetic field treatment to garden pea (*Pisum sativum* l.) seed on germination and seedling growth. *Pak. J. Bot*., 44(6): 1851-1856.

Ning, Z.H., L.Q. Xuan and K. Abdollahi. 2005. Seed coat structure of *Pinus koraiensis*. *Microscopy and Microanalysis*, 11: 1158-1159.

O'Brien, T.P., N. Feder and M.E. McCully. 1964. Polychromatic staining of plant cell walls by Toluidine blue O. http://www.cas.muohio.edu/meicenrd/ anatomy/ ch4histology.

Pandeya, D., C.S.Young, S.S.Kim, H.H. Shu and S.G. Kang. 2007. Proteome analysis of photoperiod sensitive rice mutant treated in different photoperiods. *Korean J. Crop Sci*., 52: 281-288.

Parrish, D.J. and A.C. Leopold. 1977. Transient changes during soybean imbibition. *Plant Physiol.,* 59: 1111-1115.

Qutob, D., F. Ma, C.A. Peterson, M.A. Bernards and M. Gijzen. 2008. Structural and permeability properties of the soybean seed coat. *Botany*, 86: 219-227.

Reichel, M., R. Carle, P. Sruamsiri and S. Neidhart. 2011. Changes in flavonoids and nonphenolic pigments during on-tree maturation and postharvest pericarp browning of Litchi (*Litchi chinensis* Sonn.) as shown by HPLC-MS. *J. Agric. Food Chem*., 59: 3924-3939.

Rolston, M.P. 1978. Water impermeable seed dormancy. *Bot. Rev*., 44: 365-396.

Ruzin, S.E. 1999. Plant microtechnique and microscopy. *Oxford University Press*, New York, USA.

Santana, V.M., R.A. Bradstock, M.K. Ooi, A.J. Denham, T.D. Auld and M.J. Baeza. 2010. Effects of soil temperature regimes after fire on seed dormancy and germination in six Australian Fabaceae species. *Aus. J. Bot.*, 58: 539-545.

Shao, S., C.J. Meyer, F. Ma, C.A. Peterson and M.A. Bernards. 2007. The outermost cuticle of soybean seeds: chemical composition and function during imbibition. *J. Exp. Bot.,* 58(5): 1071-1082.

Wolf, W.J., F.L. Baker and R.L. Bernard. 1981. Soybean seed-coat structural features: pits, deposits and cracks. *Scanning Electron Microscopy,* (III): 531-54.

Zamin, S.S. and M.A. Ajmal Khan. 2010. The role of seed coat phenolics on water uptake and early protein synthesis during germination of dimorphic seeds of *Halopyrum mucronatum* (L.) staph. *Pak. J. Bot*., 42(1): 227-238.

Zhou, S., H. Sekizaky, Z. Yang, S. Sawa and J. Pan. 2010. Phenolics in the seed coat of wild soybean (*Glycine soja*) and their significance for seed hardness and seed germination. *J. Agric. Food Chem*., 58: 10972-10978.

(Received for publication 10 April 2013)