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SORGHUM (SORGHUM BICOLOR (L.) MOENCH SUBSP. BICOLOR) PLANTLETS RISOGENESIS PRODUCED IN THE FIRST TWO WEEKS OF CARYOPSIS GERMINATION ON FILTER PAPER OR IN VITRO

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ABSTRACT. In this study, we used sorghum caryopsis (*Sorghum bicolor* (L.) Moench subsp. *bicolor*) to investigate the reaction process of embryo, and subsequent growth of the seedlings organs during germinaţion, as took place on filter paper or under vitroculture conditions, subjected to *natural light* or *white fluorescent light* illumination. The *root system* formation of sorghum is a complex phenomenon, and interestingly, we have found that a *red pigmentation* appears in some zones of the root. In this paper, we report a comprehensive morphological study of this process. We have found the *red pigment* to be located in the vacuolar juice of rizodermals cells or in the hypodermis, and occasionally, it is seen in some cortical parenchyma cells of radicles, or the endoderm. The identified *red pigments* are featuring characteristics like *anthocyanins*, whose roles in *root system* formation are not known.

Keywords: Sorghum, risogenesis, radilcles, lenght, pigmentation

INTRODUCTION

Sweet sorghum (*Sorghum bicolor* (L.) Moench subsp. *bicolor*) is valuable plant for farming in arid and semiarid lands of Africa, Asia and Latin America, but - more recent - this species has penetrated the Europes economy, though the production of sorghum in the European area is much lower (*Berenji* and *Dahlberg*, 2004).

In a previous study, we have reported the morpho-anatomical characteristics of sorghum caryopsis at different stages of germination (*Stana* et. al., 2011). The sorghum caryopsises are *germinating* and *growing* relatively fast that makes them suitable *biotest models* for phytophysiologysts to study the reaction of different plantlets organs to environmental factors. Our laboratory experience suggests that such *biotests* are useful to investigate the reaction of plantlets organs to different enviromental factors, such as the type and concentration of different growth regulators, placed within the germination support (*Stana* and *Chachita*, unpublished results). Moreover, such *biotests* have shown effectiveness for *allopathy*, *phytotoxicity*, and root system seedling studies in order to investigate the germination and embryo growth parameters influenced by different chemical regulators. Nevertheless, during our experiments we found that the pericarp of sorghum caryopsis and testa, in some areas (Pl.I, Fig. 1 and 2) undergoes a *red pigmentation* at the surface tissues, a trait with variable penetrance and expressivity.

Hahn and *Rooney* (1986) suggested that the *red color* of sorghum pericarp is the result of a combiation of *anthocyanic* and *anthocyanidin pigments* with other *flavonoids* compounds. In addition, *Hahn* and *Rooney* (1986) also said that *anthocyanins* are converted into corresponding *anthocyanidins*, since they are very unstable in acid medium.

Chung et. al., (1998) have subdivided the *phenolic* compounds into *phenolic acids*, *flavonoids* and *tannins*, also in the category of sorghum *flavonoids* are found *flavans*, which - if hydroxylated to C_3 - became ***anthocyanidins***. Later, *Rooney* and *Serna-Saldivar* (2000) suggested that *flavans* are ***anthocyanidins***, and most of them are *leuco**anthocyanidins***, aspect confirmed by *Waniska* (2000).

*Waniska (*2000) suggests that in the sorghum caryopsis pericarp and in testa do exist pigments such as *tannins*, compounds known as *polyphenols polymers* in a rate of approx. 2-3%. *Butler* (1990) also reported the existence of *tannins* in testa. Sometimes, the endosperm can be yellow colored. After *Hagerman* et. al., (1998), *tannins* have antioxidant properties, aspect confirmed by *Isaacson* (2005).

The presence of *anthocyans* pigmentation in the pericarp and testa of sorghum (*Sorghum bicolor* (L.) Moench subsp. *bicolor*) grain has been reported by many researchers (*Hahn* et al., 1984; *Awika* et al., 2005) However, the existence of such pigments in sorghum plantlets tissues organs, especially in their radicles, during the first days of germination seemed to be a new observation,that made us to analyze this macroscopically visible feature through electron microscopy, and a special

attention was paid to highlighten the presence of *anthocyans* in the vacuolar juice.

In the following, we describe the main results we obtained during the experiments done by us.

MATERIALS AND METHODS

Given the complexity of germination process of sweet sorghum caryopsis, we decided to perform a series of experiments that allow us to elucidate a series of aspects that occur in plantlets, during the early stages of germination.

We used sorghum (*Sorghum bicolor* (L.) Moench subsp. *bicolor*) plantlets as a *experimental model* in our research to investigate embryo germination, and the growth of plantlets organs during germination facilitated on filter paper or under vitroculture conditions (on aseptic environment), illuminated with *natural* or *fluorescent light*.

In our experiments we worled with sorghum caryopsis, obtained from Lovrin Agricultural Research and Development Station, Timis county.

Sweet sorghum caryopses were washed for 60 minutes under running tap water to clean them from any dust deposited on the pericarp. After washing, caryopses were sterilizered with a solution of 5% sodium hypochlorite (Ace) for 7 minutes, then they were washed eight times with bidistilled water, and an "*in vitro*" culture was initiated by inoculating them in culture bottles, 10 cm high, with 3 cm in diameter. Sterilized caryopsis were inoculated under sterile conditions, on *Murashige - Skoog* ½ (1962) culture medium, modified by us, suplimented with macro-and microelements, containing FeEDTA (5 mg / l), 10 g agar agar and 15 g sugar, free of vitamins and growth regulators.

Another batch of cleaned caryopsis that were not sterilized previously, was put to germinate on filter paper, soaked with distilled water, in transparent plastic boxes, 30 cm long, 15 cm wide and 10 cm high.

In the growth chamber, germination temperature was set to 22 - 23 °C, with a photoperiodicity of 16h light/day, *natural light,* or *artificial light*, emitted by *white fluorescent tubes* with a light intensity of 1300 lux.

Germination, processes was examined at macroscopic and microscopic levels, conditions, and the later included optical microscopy, electronic transmission (type FEI Tecnai 12) and scanning (type FEI Quanta 250) microscopy. The fixation of plant samples was done as follows: (1) *prefixation* for 60-90 minutes at 4 °C, with 2.7% glutaraldehyde (prepared in 0.1 M phosphate buffer, pH 7.4); (2) four consecutive washes, each for 60 minutes at 4°C; (3) a single wash in PBS 0.15 M, pH 7.4; (4) *postfixation* in osmic acid 2% solution (OsO_4), prepared in 0,15M phosphate tampon, 7,4 pH for 75-90 minutes at 4^oC (*Kay*, 1967); (5) two consecutve washes of samples in 1.5 M phosphate buffer solution, each for 15 minutes; (6) dehydration of samples in increasing concentration of 30%, 50%, for 15 min each and 70%, 80%, 30 min each 90% acetone solutions (prepared in bidistelled water) and 100% acetone baths (*Ploaie* et al., 1979), (7) the mounting of samples was obtained through the infiltration with epoxy resin 812, (8) after the polymerization of the epoxy resin, the resulted modeled blocks were sectioned with Leica LKB multramicrotome and 300nm thick sections were obtained. (9) Sections were stained with ETS (Epoxy Tissue Stain - EMS). A double contrasting of the sections was performed, by applying a samples treatment with uranyl acetate solution (for 13 minutes), afterward with led citrate solution (for 6 minutes), thickness of ultra fine samples was of 60nm (*Hayat*, 2000). (9) Microscopical examination of semi fine sections was carried out with an Olympus BX51 microscope, using a CCD camera Media Cybernetics acquisition, and the image processing was done with the software Image - Pro Plus 4.1. Ultra fine samples were examinated with FEI Tecnai 12, transmission electron microscope.

On the other hand, embryos with caryopsis that were obtained on filter paper were fixed and analyzed with a scanning electron microscope as follows: (1) samples were collected and stored at -80^0 C for 12 hours; (2) the mechanical fracture the caryopsis was carried out, (3) then samples were fixated by incubating them in a 2.7% glutaraldehyde solution (prepared in 0.1 M phosphate buffer, pH 7.4) for 2 hours; (4)four consecutive washes, each for 60 minutes at 4°C; (5) fixated samples were mounted on a microscope holde previously chilled to a temperature of 3^o C (*Goldstein*, 2003); (6) microscopic examinations were performed with FEI Quanta 250 scanning electron microscope.

Fress transversal section from the embrionary radicles tissue were put on an blade in drop of water and to see if the red pigment shifts his color in to blue we pour over the samples bicarbonate powder to change the water pH, from basic to acid.

RESULTS AND DISCUSSIONS

In this paper, as the generation of the root system occurs, we present our results regarding the phenomena captured on sorghum radicles, so that in future papers we will expose our observations done in the direction of identifying the growing process that happens to the coleoptiles and to the first leaflet.

Considering the mentioned facts, we found that the formation of the root system is complex, and during the risogenesis process, red colour pigmentation appeared on different areas of the embryonal radicle, and later on adventives roots, a trait with variable penetrance and expressivity.

We present in plane I-V the main aspects at macroscopic and microscopic level of sorghum caryopsis and plantlets, in the first 10 days of their germination on septic conditions and after 25 days of vitrocultivation, under aseptic regime.

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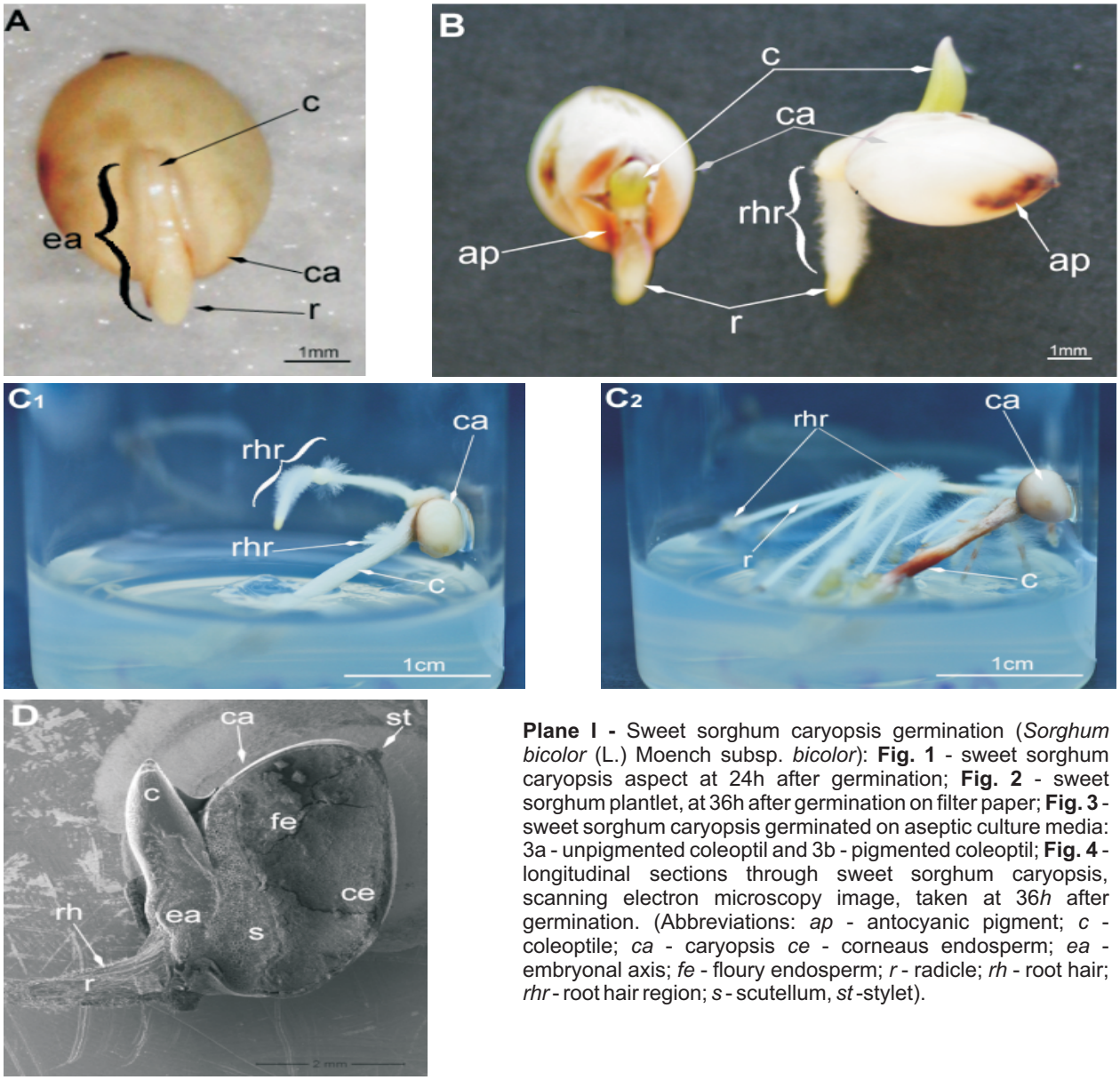
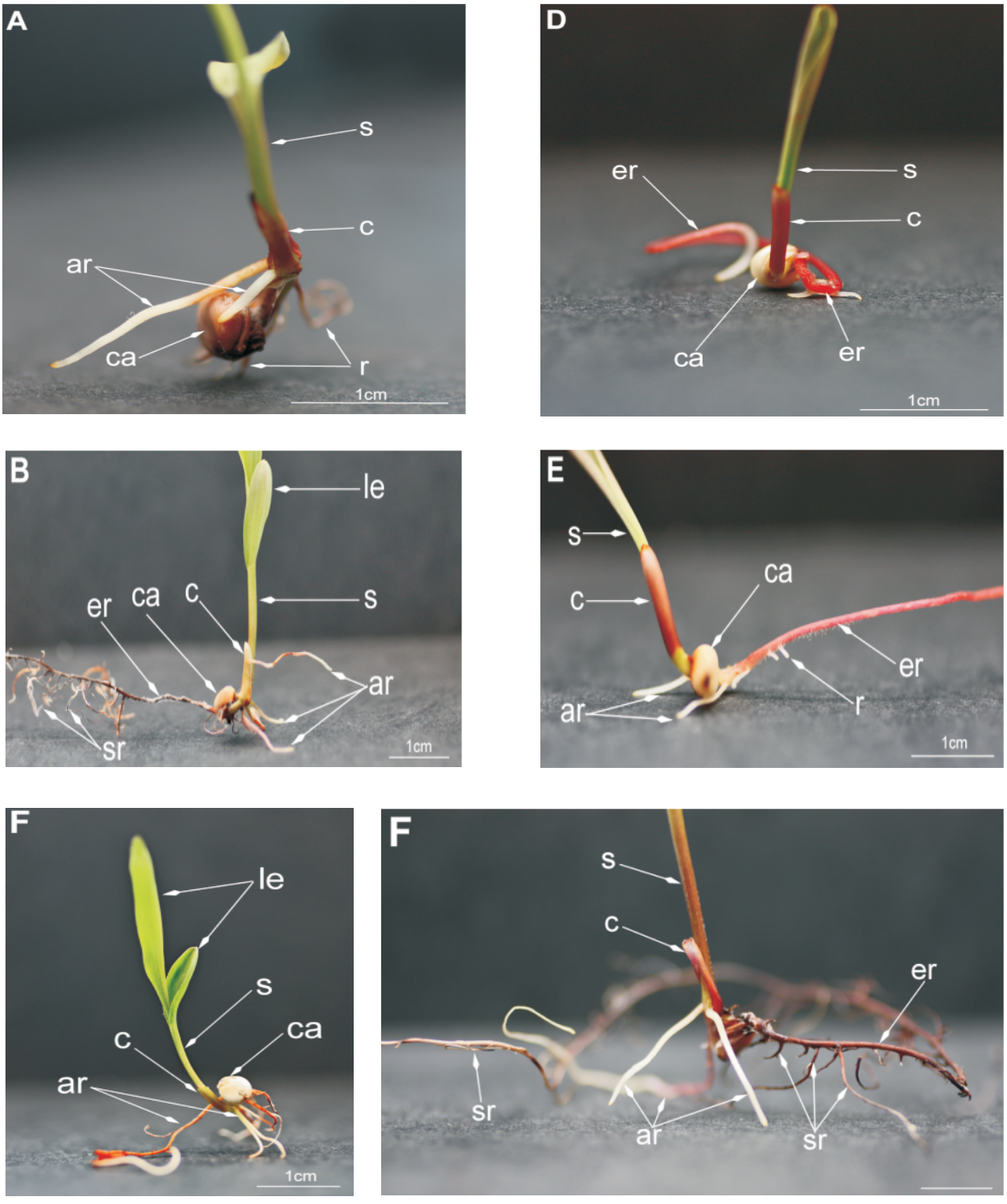
Our observations made on to sweet sorghum (Sorghum *bicolor* (L.) Moench subsp. *bicolor*) caryopsis and plantlets, at different germination stages, indicated us - but only in some plantlets - the presence of a very interesting phenomenon in their organs (roots and coleoptiles). The observed phenomenon, was natural spontaneous *pigmentation in red*, of the *radicles*, both to the *embryonary radicles* (Pl.II, Fig.1-6), as well as the *secondary* ones (Pl.III, Fig.3-4), or of the *adventives roots* (Pl.II, Fig., 2, 3 and 6), and of some *coleoptiles* (Pl.I, Fig. 3b; Pl.II, Fig. 1, 4 and 5; Pl.III, Fig. 1 and 3).Thus, in plane I-V, there are macroscopic images (Pl.I, Fig.1-3; Pl.II, Fig.1-6; Pl.III, Fig.1-4; Pl.IV, Fig.1-6), or microscopic (Pl.I, Fig.4 - scanning electron microscopy image; Pl.V, Fig.1 and 2, 4 and 5, containing images of light microscopy; while Pl.V Fig. 3 and 6 presents transmission electron microscopy images), captured mainly in the root system. Notable aspects of sorghum coleoptiles are not discussed here.

The most interesting thing that caught our attention was the presence of a *red pigmentation* in some areas of the roots or on the entire root system of sorghum (*Sorghum bicolor* (L.) Moench subsp. *bicolor*) plantlets, the variety we worked with (Pl.III, Fig. 3). Phenomenon, which alternate with the presence of - some *white colored* parts - process early highlighted, especially in *embryonary radicles* (Pl.II and III), reaction observed as the root system is forming. However, that not all the sorghum plantlets present "*bicolor*" *embryonary radicles* (Pl.I, Fig.3 and Pl.II, Fig.3), as in fact also no *secondary ones* or *adventive roots*, generated from the first internode and node, which pierce the *coleoptiles* that can be as well red colored (Pl.III, Fig. 1, 3; and other figures in Pl.II and IV). The phenomenon of radicles pigmentation, seen by us under septic and aseptic conditions as caryopsis germinated and the plantlets formed, was not uniform.



Plane I - Sweet sorghum caryopsis germination (*Sorghum bicolor* (L.) Moench subsp. *bicolor*): **Fig. 1** - sweet sorghum caryopsis aspect at 24h after germination; **Fig. 2** - sweet sorghum plantlet, at 36h after germination on filter paper; **Fig. 3** - sweet sorghum caryopsis germinated on aseptic culture media: 3a - unpigmented coleoptil and 3b - pigmented coleoptil; **Fig. 4** - longitudinal sections through sweet sorghum caryopsis, scanning electron microscopy image, taken at 36*h* after germination. (Abbreviations: *ap* - antocyanic pigment; *c* - coleoptile; *ca* - caryopsis *ce* - corneaus endosperm; *ea* - embryonal axis; *fe* - floury endosperm; *r* - radicle; *rh* - root hair; *rhr* - root hair region; *s* - scutellum, *st* -stylet).



Plane II - Sweet sorghum (*Sorghum bicolor* (L.) Moench subsp. *bicolor*) plantlet, germinated on soaked filter paper. Images taken at 36h after germination. (Abbreviations: *ar* - adventive roots; *c* - coleoptile; *ca* - caryopsis; *er* - embryonal radicle; *le* - leafleats; *r* - radicle; *rh* - root hair; *rhr* - root hair region; *s* - scutellum, *s* - stem; *sr* -- secondary roots).

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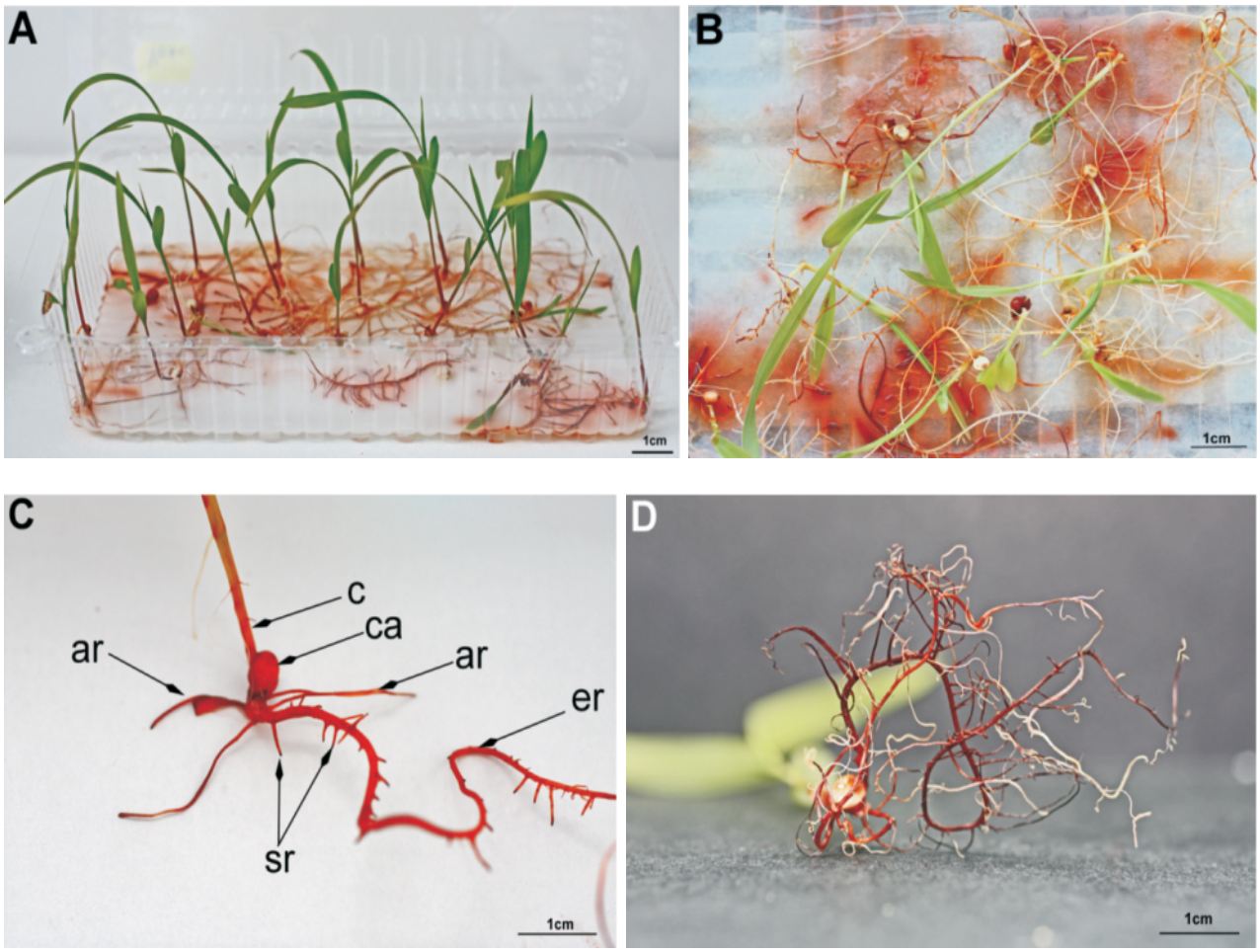
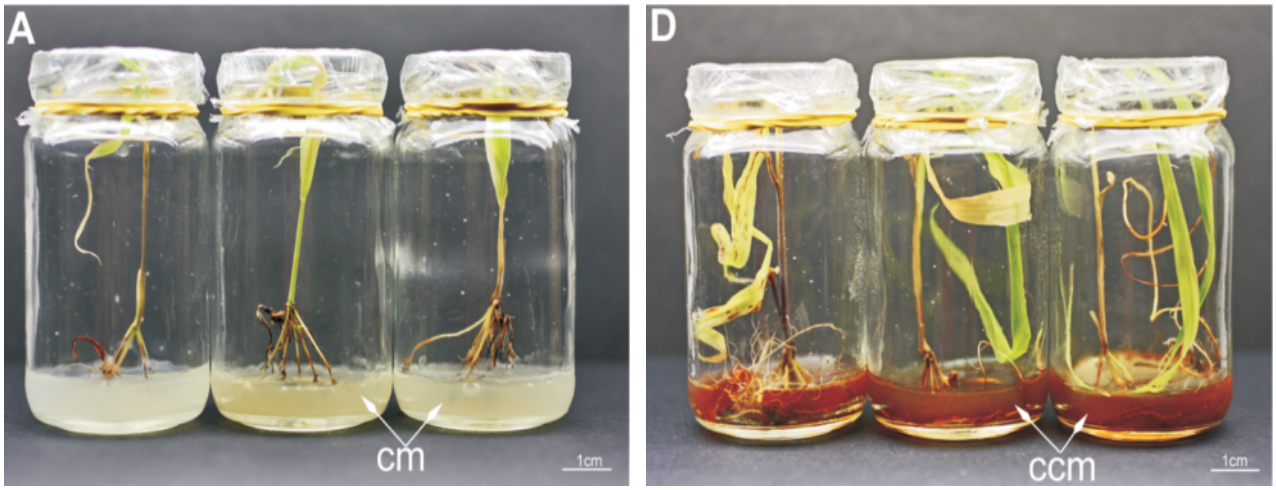
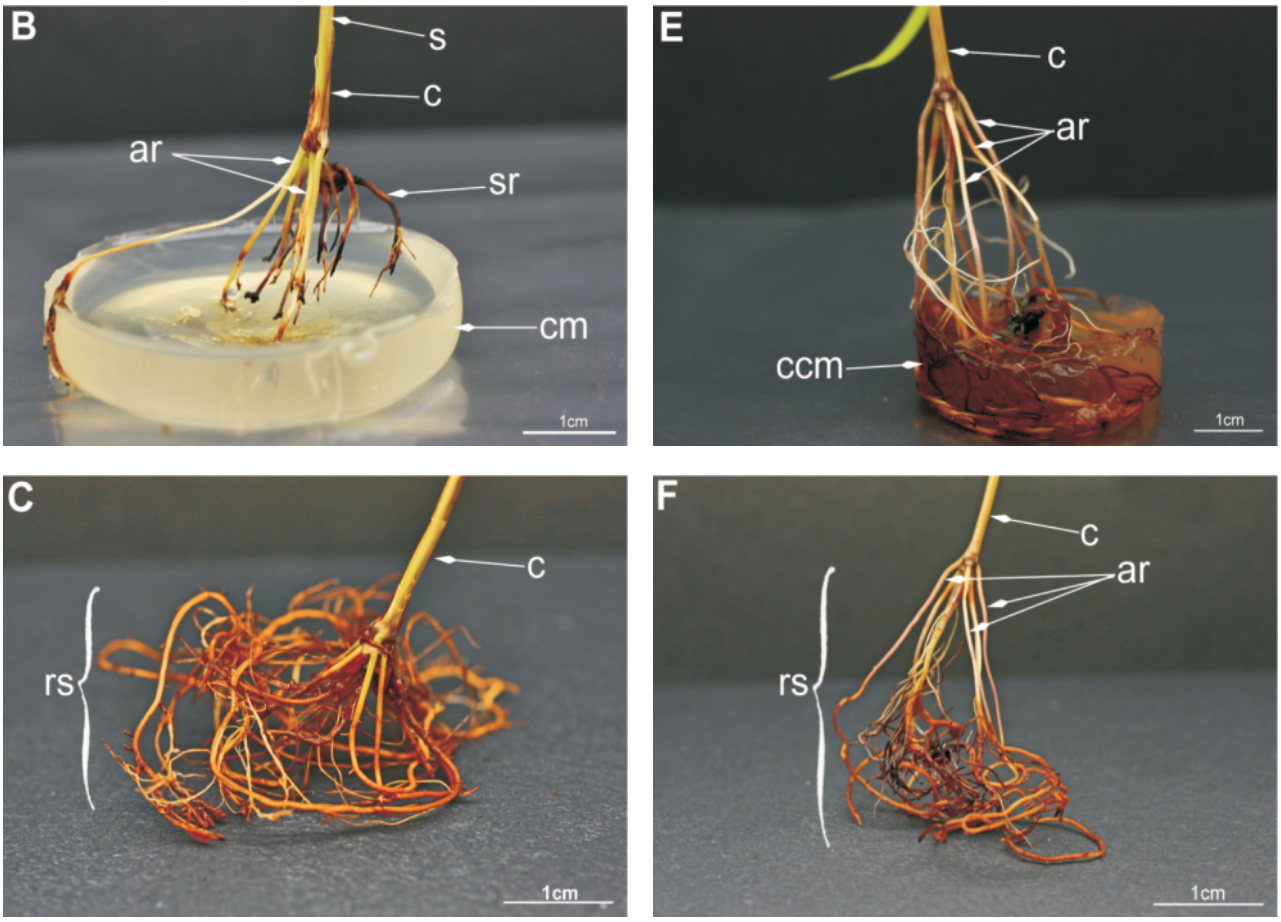
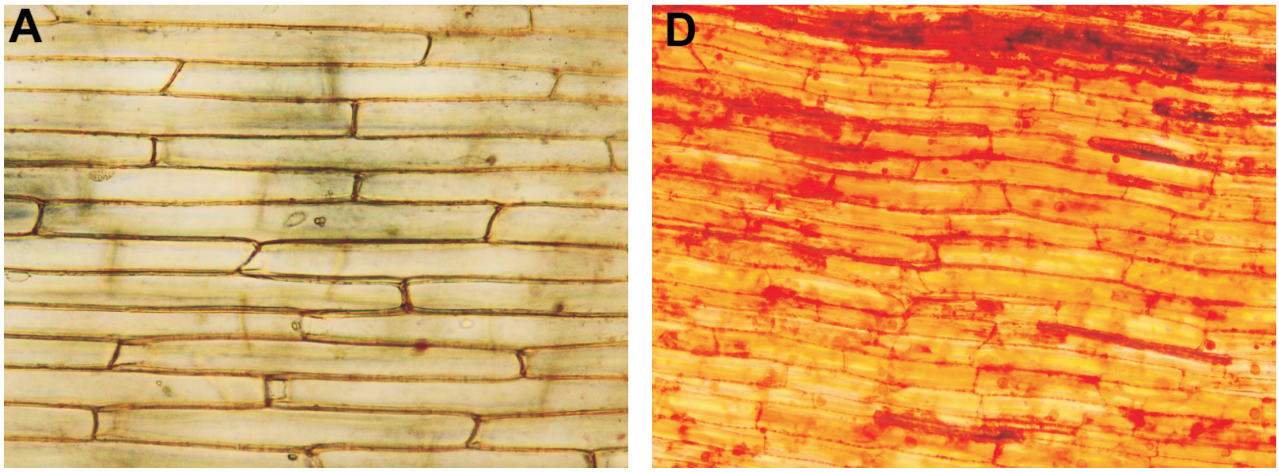


Plane III - Sweet sorghum (*Sorghum bicolor* (L.) Moench subsp. *bicolor*) plantlets, germinated on soaked filter paper. Images taken at 10 days after germination. Fig. 1 - 4, red pigmentated roots (some of them). (Abbreviations: *ar* - adventive roots; *c* - coleoptile; *ca* - caryopsis; *er* - embryonal radicle; *sr* - secondary roots).





Plane IV - Plantlet resulted from *in vitro* germination of Sweet sorghum (*Sorghum bicolor* (L.) Moench subsp. *bicolor*) caryopsis, on sterile media, substrate based on Murashige - Skoog ½ (1962) culture media. Images taken at 25 days after germination. (Abbreviations: *ar* - adventive roots; *c* - coleoptile; *cm* - culture media; *ccm* - coloured culture media; *rs* - radicular sistem; *s* - stem; *sr* - secondary roots).



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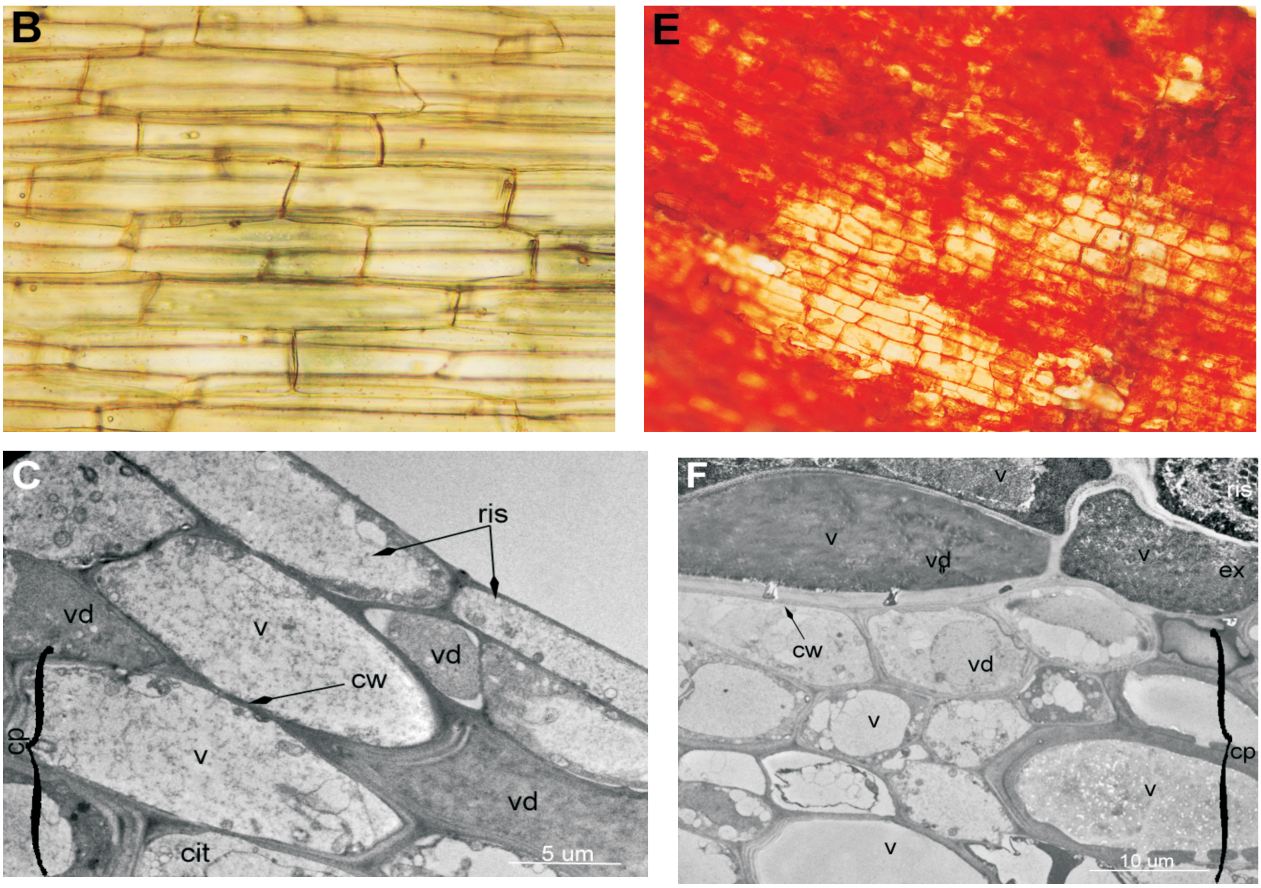

Plane V - Optical microscope images captured at sweet sorghum (*Sorghum bicolor* (L.) Moench subsp. *bicolor*) plantlets rizodermis, viewed in the six days of caryopsis germination, performed on fresh embryonal radicle (Fig. 1 and 4, ob. 2x; Fig. 2, ob. 40x and Fig. 5 ob. 10x) and transversal sections through embryonal radicle, transmission electron microscopy image (Fig. 3 and 6). (Abbreviations: *cp* - cortical parenchyma; *cw* - cell wall; *v* - vacuole; *vd* -vacuolar deposits; *ex* - exoderma, ris - risodermis).

We surprised at coleoptiles level (Pl.II compare Fig. 2 and 3 whit Fig. 1, 4, 5 and 6) the same pigmentation to sorghum caryopsis germinated "*in vitro*" (Pl. IV, Fig 1 and 4). Previously, to that mentioned we would add that not only some of the roots were red colored (Pl.II, Fig.4-6 and Pl.III, Fig.1-4), but part of this pigment - probably *anthocyans* - was desorbed outside their cells. He colored in red the filter paper on which caryopsis were germinated (Pl.III, Fig. 1 and 2) or was released in vitroculture medium (Pl.IV, Fig. 4 and 5), where germination occurred on aseptic environment.

To our knowledge, such observations have not been reported previously in the literature, and it is possible that the taxonomies have named the Sorghum *bicolor* (L.) Moench subsp. *bicolor* species and cultivars marking them with the term "*bicolor*" as feature of sorghum the plantlets with red coloring organs in natural conditions.

For a phytophysiologist or a phytomorphologist, the radicles coloring in red of some sorghum (Sorghum *bicolor* (L.) Moench subsp. *bicolor*) plantlets, is rather unusual observation. Many questions could be raised over this "duplicitary" reaction of some radicles of sorghum plantlets, manifested by the presence or absence of red pigmentation at their level. Especially since - as shall be in our other articles seen - light influence, can lead to red coloring on the surface of plantlets organs: embryonal radicles, secondary or adventives roots.

Waniska (2000) sugesst that not only caryopsis pericarp contains a red pigment (probably protoantocianidinic), but also cells from testa seed, which are masked, by starch granules present in pericarp. Earp and Rooney (1982) and Rooney and Miller (1982) categorize these as part of phenolic compounds.

All Waniska (2000) highlighted the fact that all species or varieties of sorghum contain *phenols* and *flavonoids*. Those that produce *condensed polyphenols* or *condensed tannins* have pigmented testa (*Butler*, 1990). All of these are considered as *protoantocianidine*, which presents the highest concentrations of *phenolic* compounds (*Dykes* et al., 2005).

The phytobiological role of *anthocyans* and the anatomical location of pigmentation in plant cells are controversial (*Lrev-Yadun* and *Gould,* 2009). *Quine* et. al., (2009) suggests that anthocyanins absorb visible light, even the ultraviolet one, and therefore they play a protective, antioxidant role, by protecting plantlets cell from photooxidative stress. Further studies are conducted in our laboratory, to elucidate the presumptive antioxidant role of such red pigments during the embryonic root system formation.

In the case of sorghum, in the same experimental group, there was great heterogeneity of the radicles response, either embryonal radicles, secondary (first - order) or adventive roots (Pl.II, Fig. 1-2; Pl . III, Fig.1-6). Under vitroculture conditions (Pl.IV)(as, indeed, so in nature) roots of some plantlets release compounds in substrate, that change the color of the culture medium, in case of sorghum could be - we suggest - *anthocyanins* (Fig. 1 and 3). In time, they can be oxidized, gaining a brown color (Pl.III, Fig. 1-4); in case of grains germinated on filter paper (Pl.II, Fig.3), the color turns red in the right of roots on this type of substrate

The embryonal radicles risodermis not featuring pigmented areas, visualized by optical microscope, are illustrated in figures 1 and 2 from plate V while in the figures 4 and 5 are shown various images with red pigmented embryonal radicles. In figures 4 and 5 (Pl. V) images capture aspects seen in the optical microscope. Figures 3 and 6 are showing transmission electron microscopy images, captured in an area less colored root (Pl. V, Fig. 3 and 4). In these samples, located in the deeper layers of roots, respectively in their cortical parenchyma we can see fine electrondense, vacuolar deposits. In figures 5 and 6 (Pl.V) red coloration, mainly, was present at the risodermas or hypodermas, level (Fig. 6, Pl.V).

Yet we could not it identify the nature of vacuolar deposits, but we found that red pigment color - visible macroscopic and microscopic at optical microscope - turns blue if the pH of water in which the samples are placed it passes from acid to base, with addition of bicarbonate powder. The change in color from red to blue of the vacuolar juice suggests that the vacuolar pigment could be of an anthocyanin type. We suggest that, especially in the case of those rizodermale cells, which are not red colored, the presence in the vacuolar juice of leucoantocians, compounds that are not colored. Therefore, endocelular reactions that occur in cells that undergo a red pigmentation in their vacuolar juice are still unknown; the phenomenon was not described yet in the literature. Perhaps in the near future, cellular and molecular biology research will help elucidate this phenomenon.

CONCLUSIONS

Sweet sorghum (Sorghum *bicolor* (L.) Moench subsp. *bicolor*) plantlet, resulted from caryopsis germination either on filter paper placed in plastic container, regular humidified with bidistilled water, either in vitro on Murashige - Skoog (1962) agarized, culture medium (without vitamins and growth regulators), presents an intense red pigmentation process. Pigmentation occurred on embryonal radicles and sometimes on - first order - secondary roots, or on adventive ones, neoformate on first intrenod track or on stemlet node.

Nevertheless, at the same sorghum plantlets, or on the same type of roots, in identical experimental conditions, they can appear unpigmented. It is difficult to explain this phenomenon, especially since this process is with variable penetrance and expressivity. Sometimes, the root system of plantlets can be - all - red pigmented. Often, the secondary roots and the adventive one can be white, while others can be red colored. However, adventive roots can be white or red pigmented, even when the plantlets coleoptiles are green and pierced by them.

The red pigment considered by us as anthocyanins type, is located in the vacuolar juice of the rizodermal cells or can sometimes be it found in the hypodermis or, occasionally, may be present in some cortical parenchyma cells of roots, or even in the endoderma.

Electron microscopic examination revealed that the vacuolar juice with anthocyans undergoes a fine granulation of their vacuolar content; witch can be a result of samples preparation with osmic acid. It is not clear if leucoantocians accumulated in vacuolele cells of root tissue undergo this fine granulation, or only those with red anthocyanins. Future researches are needed to elucidate this phenomenon.

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