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Full Length Research Paper

Effects of early water stress levels on berry set and berry development in Merlot cv. (*Vitis vinifera* L.)

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Early water deficit at flowering results in poor berry set or aborted grape yield reduction. The main aim of this study was to find out the effects of water stress levels on berry set and embryo berry development. This study was carried out in Montpellier using by ECOTRON System, SupAgro/INRA, France. Seven years old Merlot / SO_4 grafting combination was used as a plant material. Potted grapevines were grown under natural conditions in open field. A randomized block design was used with 3 replications and each parcel had 2 grapevines and 4 water stress levels. Stress Levels were WS_0 (control) 0; -0.2 MPa, WS_1 -0.2; -0.4 MPa, WS_2 -0.4; -0.6 MPa, and WS_3 -0.6; -0.8 MPa, respectively. The stress period was started in the second week of May in the 17th E-L stage and ended in the second week of June in 27th E-L stage. Flower samples from clusters were collected in three days intervals and samples were examined by Technovit method. Under this early water stress conditions, the pollen viability and pollen germination ratios were not affected, but berry set ratio, phenologic stages and berry development were negatively affected. It was determined in Merlot cv. early water stress (between 19th to 29th stages) should be avoided especially when Ψ_{pd} values decrease below -0.4MPa

Key words: *Vitis vinifera* L., leaf water potential, embryo development, berry set, Merlot cv.

INTRODUCTION

Winegrape quality is determined by berry composition (Coombe, 1990). For that reason, flowering and berry set are critical stages in grapevine development (Longbottom, 2007). In budburst to flowering: avoid water stress. During this period, rapid shoot growth ensures the development of an adequate leaf canopy to sustain production. In flowering to berry set stages, high water stress at flowering can result in poor fruit set (hen and chicken), aborted fruit (shot berries) or smaller berries, all of which reduces yield. B berry set to veraison stage, the vine is less susceptible to moderate water deficits. (Ward, 2010).

Berry size and water content are two of the main factors affecting berry growth and wine quality that are influenced by irrigation (Girona et al., 2006). It is often stated that reproductive growth and thus yield is less sensitive to water deficit than is vegetative growth. However, the impact of water stress on yield depends on when the stress occurs. Early on (i.e. post-budbreak) the developing flower clusters can compete quite successfully with the growing shoots for limited water (Keller, 2004; Bahar et al., 2011b). Water deficits between anthesis and verasion decreased berry size (Ojeda et al., 2001). Water stress at flowering may result in poor berry set or aborted grape yield reduction. Early season described budburst to flowering and flowering to berry-set. When combined, these stages utilize approximately 14% of the annual water requirement (Chalmers, 2009).

It is well known that Merlot cultivar is highly sensitive to coulure. For decades, researchers have experimented and discussed parameters which are considered to prevent grapevine from the shattering (coulure) such as; topping, pinching, cincturing, water availability, N fertilizing, hormones, foliar sprays (Zeftawi and Weste, 1970; Bessis and Fournioux, 1992; Delas et al., 1991; Bentchikou et al., 1992; Bessis, 1993; Delas, 1994;

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Bahar and Yasasin, 2010; Kok, 2011). In addition to this parameters, the followings; climatic factors, environment-sensible stages of the floral development, disfunctions of the flower structures (polen and embryo malformations), nutrition and water supply, source-sink relationships, etc. are postulated as factors having impact on coulure and berry set (Carbonneau and Ollat, 1993). Although, physiology of coulure and its sources are studied for years, effects of high stress conditions on berry set and embryo (berry) development are still not well-known and need to be searched.

Hardie and Considine (1976) report that the severe water stress is induced in container-grown grapevines during five stages of fruit growth. Stress at each stage reduces fresh berry yield. During the first three weeks after flowering, losses are greatest and are primarily attributable to reduced berry set. Thereafter loss is associated with reduced berry size and, following stress after veraison, the failure of fruit to mature. Fruit which failed to mature also has a lower skin pigment content, whether assessed on a per-berry or per-unit-surface area basis. All berry from stressed vines is late to mature, though the delay is greatest for berry stressed during the lag phase. In addition, Bahar et al. (2011a) reported that the sudden and extreme water stress resulted in smaller berries and lead to an increase of anthocyanin concentration, pH, FCI and PTI values at harvest. On the contrary there is a reduction in the values of 100 berry weight, berry volume, TSS, sugar concentration, sugar per berry, K and tartaric acid. In conclusion, extreme water stress therefore has a negative effect on berry quality. If water deficit is applied early in the season, the effects will be achieved mostly through a reduction of berry cell division (McCarthy, 2002); while if water deficit is imposed at later stages, the major effect will be an inhibition of berry growth (Williams and Matthews, 1990).

The effects of early and late water deficits (anthesis to maturity) on pericarp cell division and enlargement of Syrah berries is determined by DNA extraction and quantification. DNA extraction profiles show that water deficit does not affect cell division. Reduction of berry size and berry weight is caused exclusively by a decrease of pericarp volume. Decreased cell volume as a result of an early water deficit from flowering to veraison is irreversible (Ojeda et al., 2001). Flowering and berry set are very sensitive to water stress as cell division is involved. It is a period when shoot growth is rapid and as a precaution, irrigation water should be applied prior to flowering if soil water levels are low (Anonymous, 2010). The main aim of this study was to find out effects of three early water stress levels on berry set and embryo (berry) development.

MATERIALS AND METHODS

This research was carried out in Montpellier in 2008 vegetation period using by ECOTRON System, SupAgro/INRA, France. Seven years old Merlot / SO_4 was used as a plant material. Potted grapevines were grown under natural conditions in open field, with a volume of 70 L for individual vine. The pots were isolated from rainfall, had a controlled drainage system, and the growing medium was a mixing of coarse sand and perlite. A randomized block design was used with 3 replications and each parcel had 2 grapevines and 4 water stress levels (WS_0, WS_1, WS_2, WS_3). In the trial, totally 24 grapevines were used. Stress levels were WS_0 (control) 0; -0.2 MPa, WS_1 -0.2; -0.4 MPa, WS_2 -0.4; -0.6 MPa and WS_3 -0.6; -0.8 MPa, respectively. At the beginning of the trial (18th of May), all shoots and clusters were counted. Taking into consideration of physiological balance, minimum rule was used for the grapevines, therefore right (2 to 4 nodes) and left branches (7 to 8 nodes) have equal number of shoots (11 to 13 shoots) and clusters (~28).

The stressed period was started in the second week of May in 17th E-L stage and stopped in the second week of June in 31st E-L stage (Eichhorn and Lorenz, 1977). Stressed vine were well watered (4 L day^{-1}) WS_0 as a control, WS_1 (3 L day^{-1}), WS_2 (2 L day^{-1}), and WS_3 (1 L day^{-1}), respectively. Drip fert-irrigation was applied four times in a day at 11:00 a.m., 16:00 p.m., 23:00 p.m. and 04:00 a.m. Plant water stress levels [as Predawn Leaf Water Potential (Ψ_{pd})] were measured at three days intervals by Scholander Pressure Chamber at 03:00 a.m., each leaf measured (Ψ_{pd}) by parcel based (Scholander et al., 1965).

Flower samples from clusters were collected in three days intervals (between 17.05.2008 and 16.06.2008) and fixed in FAA solution (Korkutal and Celik, 2007). On the purpose of explaining embryo development phases, samples were prepared by Technovit Method. Subsequently, 5 μM sections were cut by Leica RM 2165 Microtome, equipped with a glass knife. For histological evaluation, sections were stained with 0.5% Toluidine blue. Fixed preparatives were examined and photographed by Olympus BX51 microscope.

Furthermore, in the second part of the experiment; pollen viability (%) determined by aceto-carmine staining method (Marasali, 1992; Korkutal et al., 2004; Dane and Meric, 2005) and pollen germination (%) determined by agar plate method (1% agar + 15% sucrose) (Derin and Eti, 2001; Korkutal et al., 2004). Polen viability and germination ratio were mounted by Leitz DMRB Fluorescence microscope.

RESULTS AND DISCUSSION

Predawn leaf water potential (-MPa)

WS_0 as control, was fully irrigated (4 L day^{-1}). The data show that the Ψ_{pd} values were between -0.18 and -0.26 MPa in WS_0. In WS_1, Ψ_{pd} values ranged from -0.22 to -0.53 MPa. The highest Ψ_{pd} in WS_2 was -0.23 MPa, the lowest Ψ_{pd} was -0.55 MPa. The results showed that the lowest Ψ_{pd} in WS_3 was -0.78 MPa (Figure 1).

Pollen viability

It was found out that the pollen viabilitiy ratios were between 89.96 to 91.63% . Based on data which were shown in Figure 2, maximum pollen viability was observed in WS_3. There were no differences found in pollen viabilities between water stress levels.

Pollen germination

In vitro polen germination results showed that the Merlot

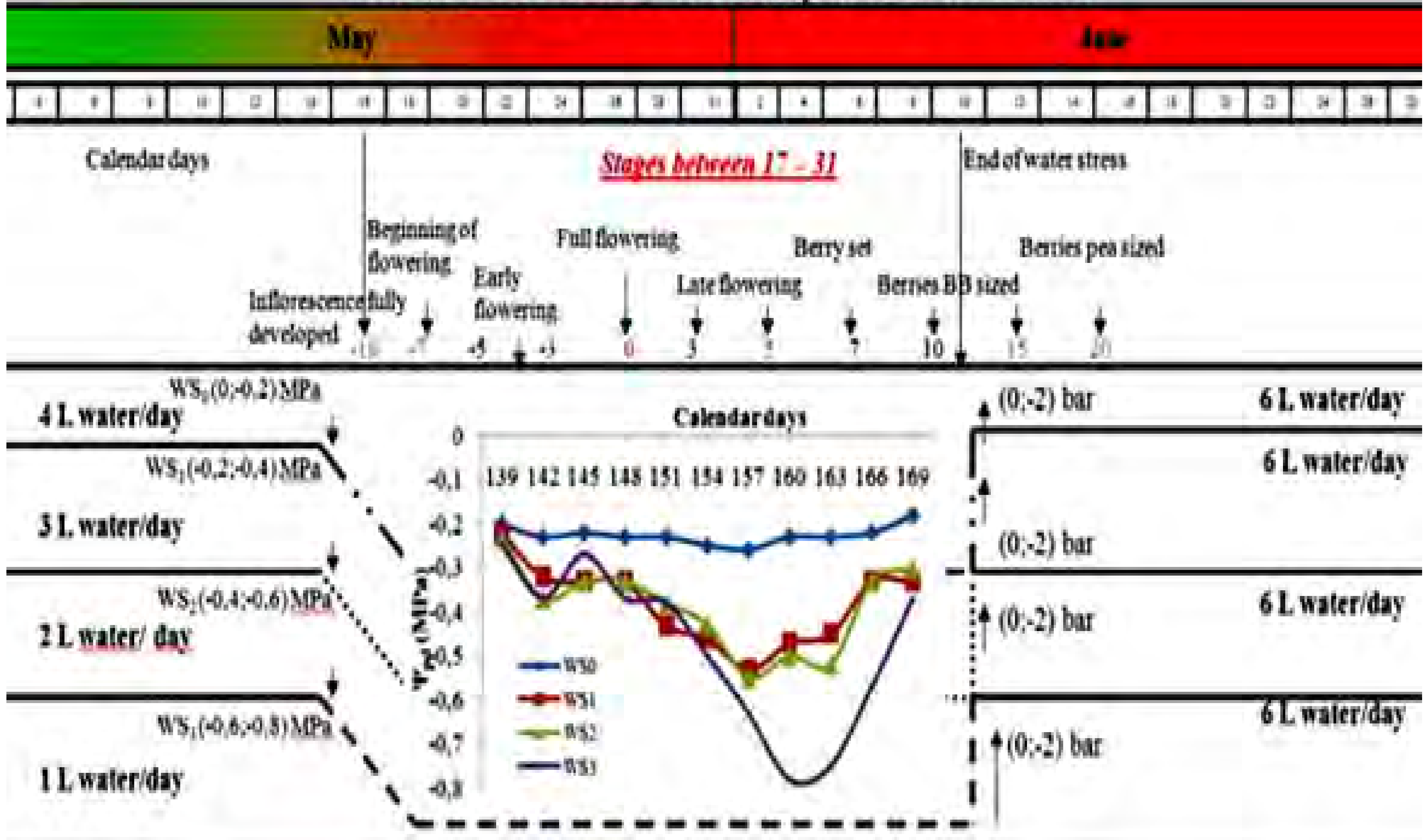

Figure 1. Water stress application scheme.

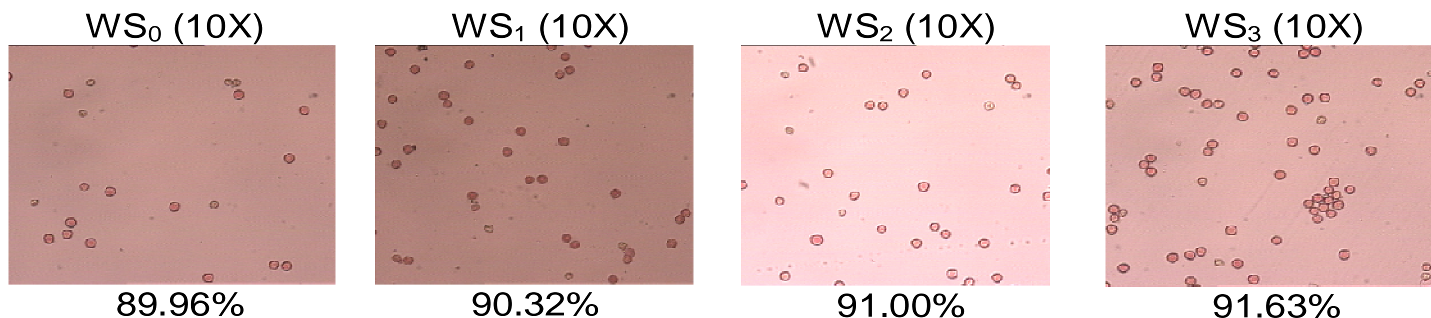

Figure 2. Pollen viability (%).

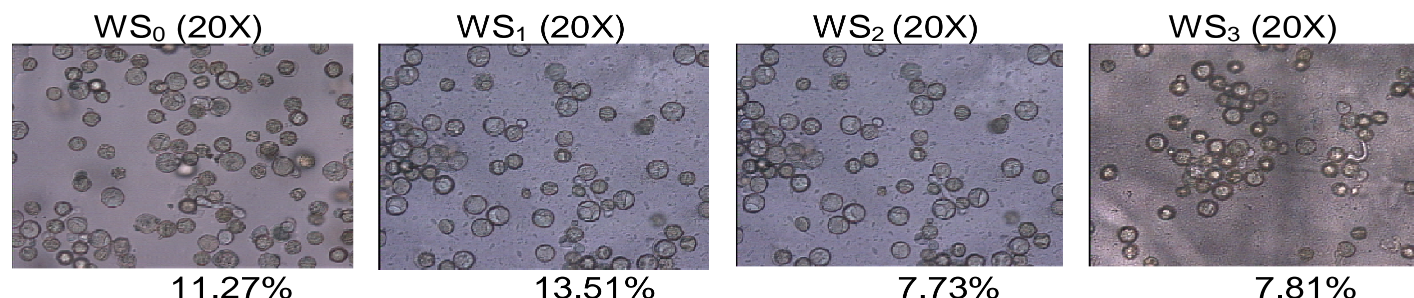

Figure 3. Pollen germination (%).

Table 1. Phenologic stages (Eichhorn and Lorenz, 1977).

Stage	Eichhorn - Lorenz	WS_0	WS_1	WS_2	WS_3
17th	Inflorescence full developed	20.05.2008	20.05.2008	20.05.2008	17.05.2008
19th	Beginning of flowering	22.05.2008	20.05.2008	20.05.2008	20.05.2008
20th	10% caps off	23.05.2008	23.05.2008	21.05.2008	21.05.2008
21th	30% caps off	24.05.2008	23.05.2008	22.05.2008	22.05.2008
23th	50% caps off (full flowering)	28.05.2008	24.05.2008	24.05.2008	23.05.2008
25th	80% caps off (late flowering)	31.05.2008	28.05.2008	28.05.2008	24.05.2008
26th	Cap-fall complete	01.06.2008	30.05.2008	28.05.2008	28.05.2008
27th	Fruit set (2mm)	06.06.2008	30.05.2008	28.05.2008	09.06.2008
29th	Berries lead-shot size (4mm)	09.06.2008	09.06.2008	06.06.2008	13.06.2008
31th	Berries pea sized (7mm)	13.06.2008	13.06.2008	15.06.2008	15.06.2008

cv. had 7.73%-13.51% values. High polen germination rate was 13.51% in WS_1 (Figure 3).

Berry set ratio

Berry set ratios were defined as 44.38% in WS_0, 38.93% in WS_1, 32.83% in WS_2 and 24.96% in WS_3 respectively, affected by the early water deficit. These results reveal that the water stress at flowering may result in poor fruit set or aborted fruit yield reduction (Chalmers, 2009). Same results were found in our study.

Phenologic stages

Ojeda et al. (1999) note that the berry development in vines grown in Ecotron and in the vineyard is congruent. Phenologic stages were affected by water stress. In WS_3, cap-fall complete stage (26th), was longer than the others. After this stage, dates of 27th, 29th and 31st stages were it took 11 days to the other water stress applications (Table 1). Moreover, as shown by the data presented in Figure 4a, inflorescence was fully developed. There were not any differences in stage 17th for flower growing between stress levels (Figure 4a).

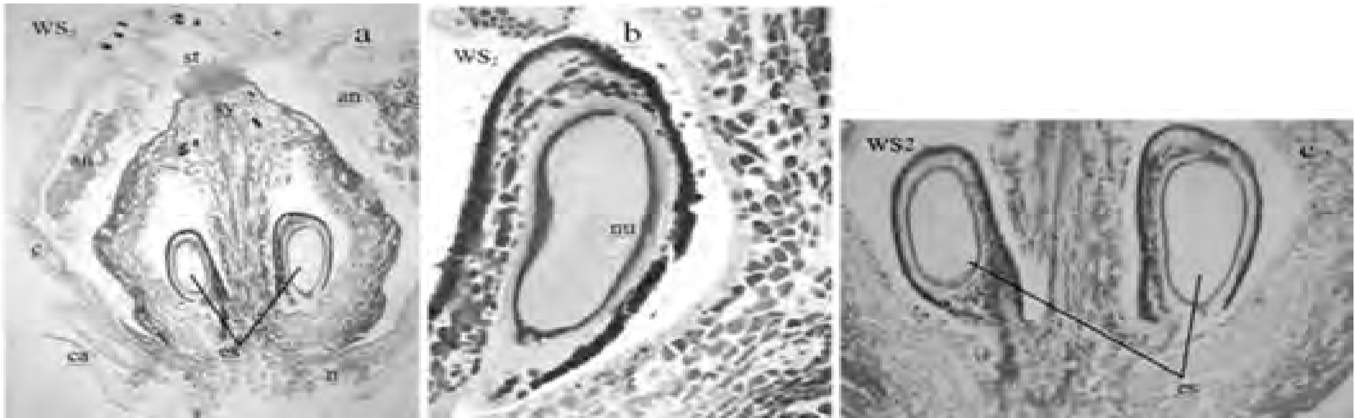

Figure 4. First samples in all stress groups were in inflorescence full developed stage (17th E-L stage). It was seen in cross section of WS_2 (a) (X4), anatrop type ovule (b) (X20), and two ovules (c) (X10). *st*, Stigma; *sy*, stylus; *ca*, calyx; *c*, corolla; *an*, anther; *es*, embryo sac; *nu*, nucellus; *mmc*, megaspore mother cell; *m*, micropyle.

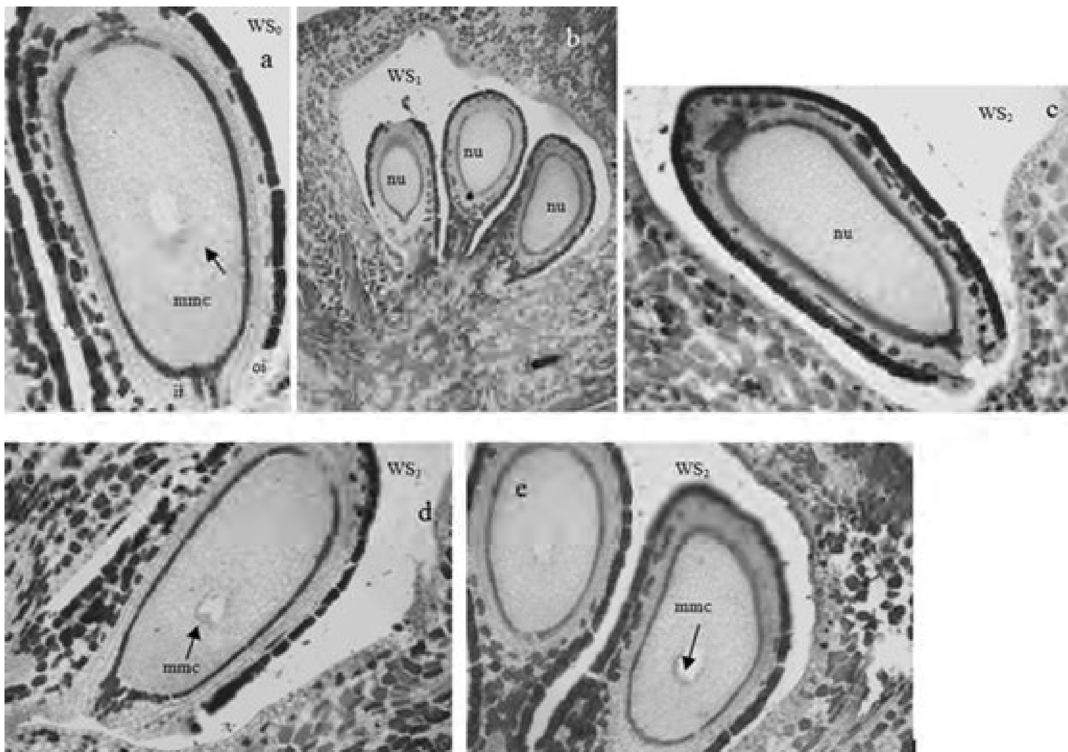

Figure 5. There was megaspore mother cell in all water stress levels in second sampling date (19th E-L Stage). Nucellus occurred in WS_0 (a) (X20), in WS_3 (d) (X20), in WS_2 (e) (X20), in WS_1 (b) (X10) in WS_2 and (c) (X20) in WS_2.. WS_1 had (b) 3 embryo sacs. *mmc*, Megaspore mother cell; *nu*, nucellus; *ii*, inner integument; *oi*; outer integument.

Embryo sacs were normal (Figure 4b and 4c). Floral cap structure was attached to each flower (Poupin et al., 2007). Embryo sacs were encased in nucellus cells and anatrop types (Pratt, 1971), embryo sacs were differentiated. Inner and outer integument cells were fully developed, and were about to close to micropile aperture. As reported by Lebon et al. (2004), starch is abundant in ovary wall in Stage 17th according to E-L (1977).

Furthermore, in this stage, it was seen that the megasporogenesis phase would start soon During this stage, in the other groups except WS_0 Ψ_{pd} values were between -0.3 and -0.4 MPa (mild to moderate water

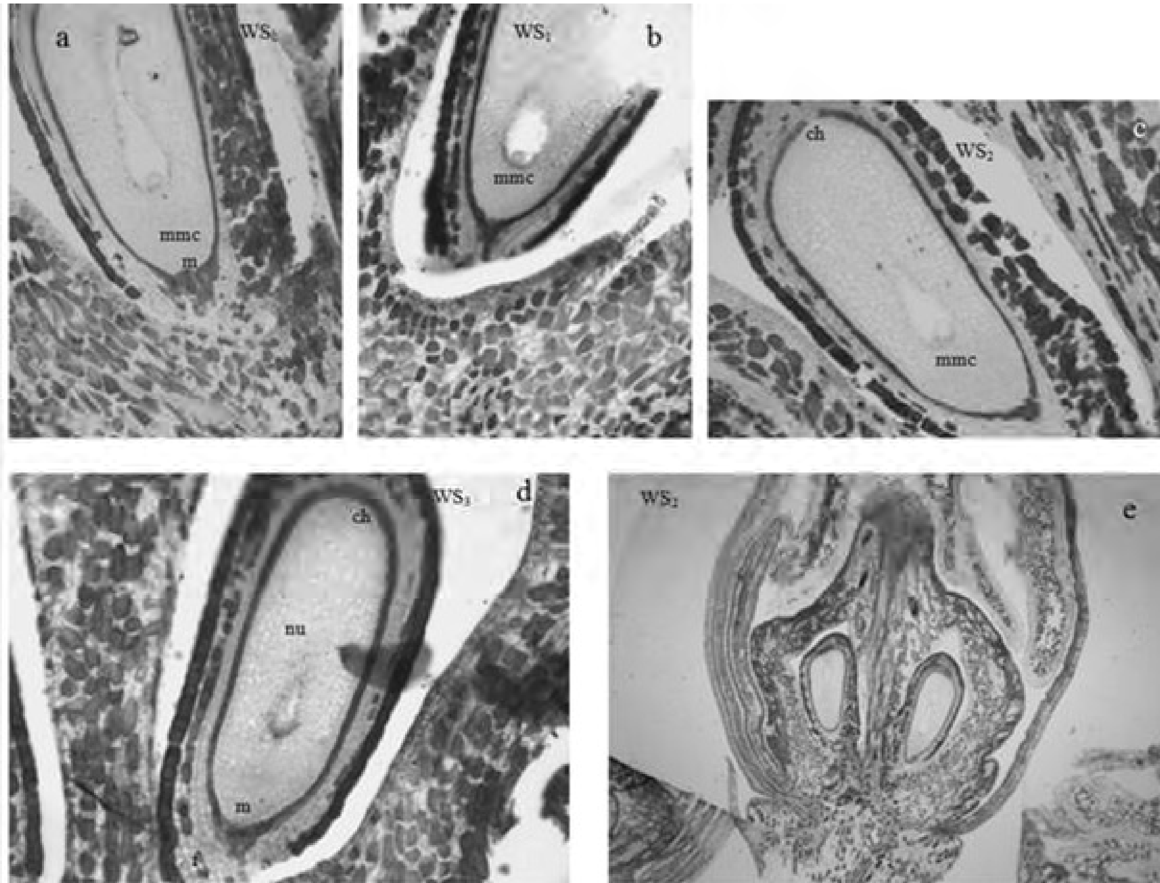

Figure 6. Megaspor mother cell was seen in early flowering stage, in longitudinal sections at 3rd sampling time (21st E-L Stage) in all WS_0 (a) (X20), WS_1 (b) (X20), WS_2 (c) (X20), WS_3 (d) (X20), WS_2 (e) (X4). *m*, Micropyle; *ch*, chalaza; *f*, funiculus.

stress).. In Figure 5, all stress groups in exception of WS_1, had megaspore mother cell in the center of embryo sacs. There were three embryo sacs in WS_1 (Figure 5b). It was normally, because Merlot variety had 2-3 seeds in berry. Generally ovary is two carpelled, in each carpel generally two ovules occure. In the mean time, some samples in the same carpel 3 ovules are discovered Marasali (2002) similar to our study. It could be said that in this stage flowers were tolerant to mid-moderate stress in Merlot cv. It is known that grapevine embryo sac is crassinucellate (=crassinucellar) type; an ovule with one or more layers of cells outside the embryo sac but distinct from the epidermis of the ovule (Maheshwari, 1950; Unal, 2006). There were apertures formed in center of embryo sacs (Figure 6a to d). Enlargement of the embryo sac leads to the destruction of most of the nucellus during its development. Polygonum type embryo sacs originate from a single chalazally located megaspore that undergoes three successive mitotic divisions. During the first meiotic division, the spindle is oriented paralel to the micropylar-chalazal axis of the nucellus. Wall formation occurs perpendicular to this axis, creating dyad of megaspores. Frequently, the dyad cell closest to the micropyle degenerates without undergoing a second meiotic division. The three non functional megaspores degenerate and are eventually crushed by the expanding functional megaspore. More also, there were tetrahedral arrangements of megaspores as in earlier reports (Maheshwari, 1950; Reiser and Fischer, 1993; Korkutal, 1999; Unal, 2006). At the same time flowers were not open yet (Figure 6e), Fourth sampling time was full flowering stage according to E-L (Eichhorn and Lorenz, 1977). But all flowers were not open in this stage and Ψ_{pd} values were still between -0.3 and -0.4 MPa. During 7-8 days Ψ_{pd} values were between these values. After chalazal megaspore is divided 3 times subsequently, there are embryo sac which has one egg cell, two synergids, three antipodal cells, and central cell that contain two nuclei (polar cell). Egg cell is located at the micropylar end of embryo sac and ultimately fuses with a sperm nucleus to produce a zygote (Pratt, 1971; Reiser and Fischer, 1993) Zygote formation was as the same as our study (Figure 7a, b, c, d). The synergids, which are located on either side of the egg cell, play an important role in fertilization The same synergids were seen in our study (Figure 7a). The pollen tube discharges its contents

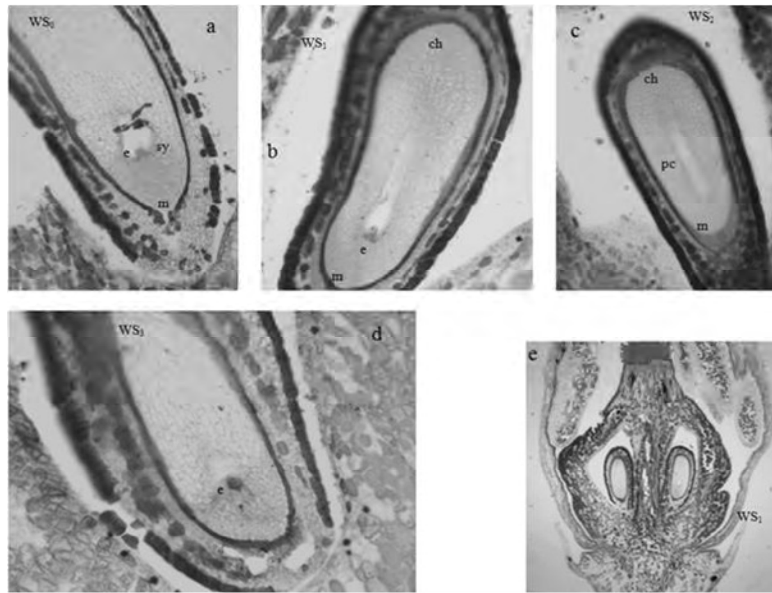

Figure 7. Sampling time 4th (23rd E-L Stage) chalazal megaspore was divided 3 times by mitosis. 2 synergid cells and egg mother cell in WS_0 (a) (X20), egg mother cell in WS_1 (b) (X20), polar cells in WS_2 (c) (X20), egg mother cell in WS_3 (d) (X20). Flowers were not open in WS_1 at sampling time (e) (X4). *e*, Egg cell; *m*, micropyle; *ch*, chalaza; *pc*, polar cell; *sy*, synergids.

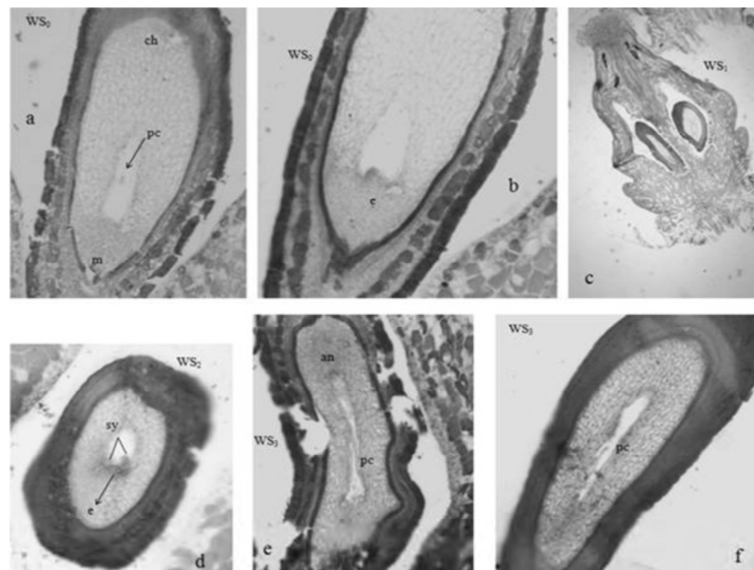

Figure 8. This sampling time (5th) was called late flowering (25th E-L Stage). Polar cell (a) (X20), egg cell (b) (X20) in WS_0, flowers opened in WS_1 (c) (X4), synergid cells in WS_2 (d) (X20), polar cell in WS_3 (e) (X20) and polar cell in WS_3 (f) (X20). *e*, Egg cell; *pc*, polar cell; *sy*, synergids; *an*, antipodal cell; *m*, micropyle; *ch*, chalaza.

in to one of the synergids prior to incorporation of the sperm nuclei into the egg and central cells (polar cells). In this stage, 3 antipodal cells existed but in microscopic sections these were not observed. It may be due to they were removed during the fixation process., Cap was still attached to some flowers (Figure 7e).

In late flowering stage, an early seed structure began to develop in all stress levels. Ψ_{pd} values reached -0.3 and -0.5 MPa (moderate to severe water stress). Polar cells for coalescent through the center of the embryo sac

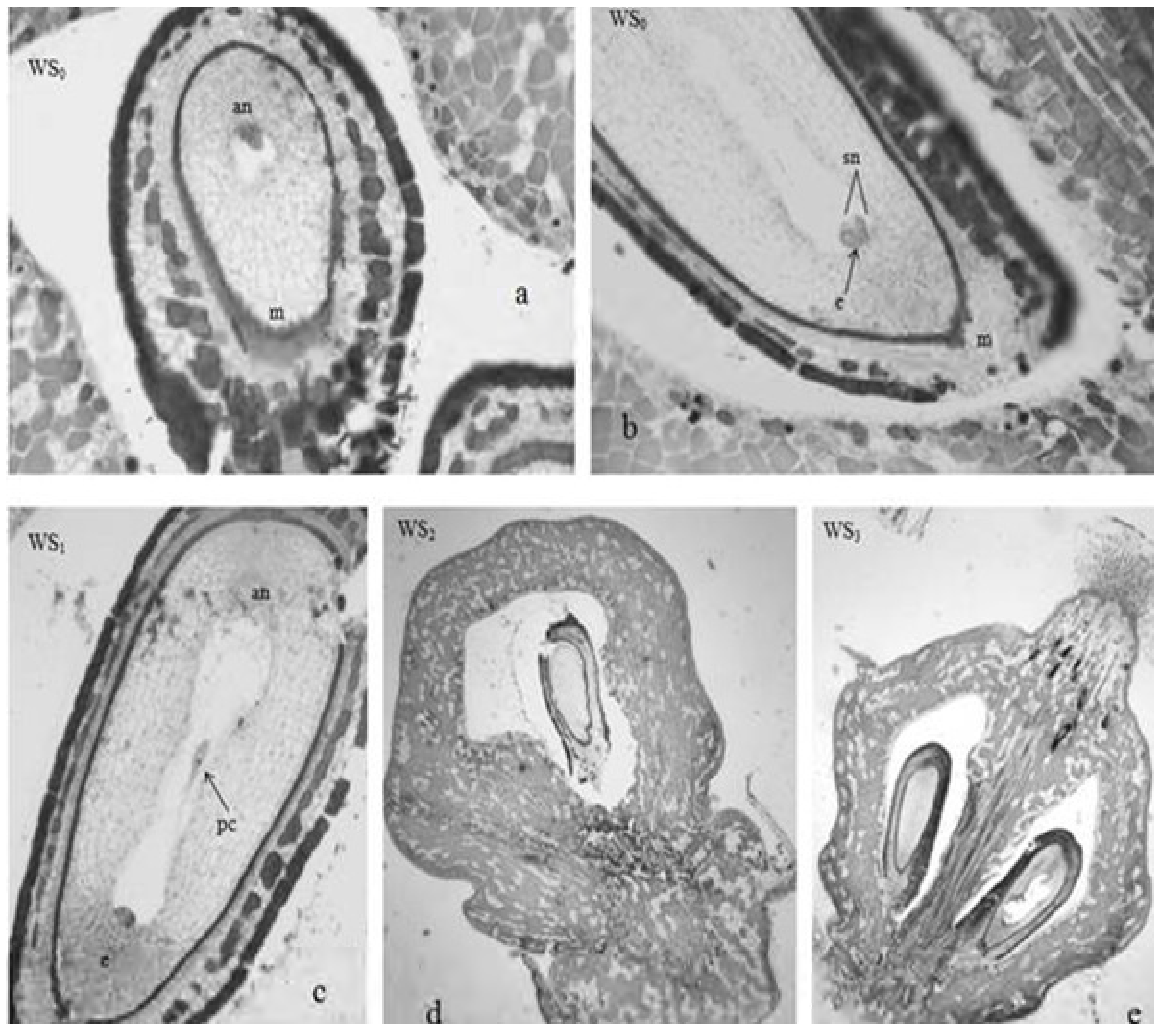

Figure 9. Sample 6 (25th E-L Stage), some of flowers were ready to fertilization, WS_0 (a) (X20), WS_0 (b) (X20), WS_1 (c) (X20), some of them were fertilized, WS_2 (d) (X4) and WS_3 (e) (X4). *an*, Antipod cells; *m*, micropyle; *sn*, synergids; *pc*, polar cells; *e*, egg cell.

(Figure 8a, e, f). Three antipodal cells were located opposite the egg at the chalazal end of the embryo sac (Figure 8e). No specific function during reproduction has been attributed to the antipodals, but they are thought to be involved the import of nutrients to the embryo sac (Reiser and Fischer, 1993; Korkutal, 1999). As shown in Figure 8b, egg cell ready for fertilization, the cap has detached from the flower and fallen away (Figure 8c). In seed development for all stress groups no differences were determined. Fusion of male and female gametes is known as fertilization and results in the production of a zygote (n + n = 2n). The second male gamete fuses with the two polar nuclei and forms a triple-fusion nucleus, called primary endosperm nucleus (2n + n = 3n) (endosperm primer cell). This two types of fusion syngamy and triple fusion take a place in an embryo sac, and the process is termed as double fertilization (Mullins et al., 1992). WS_0 and WS_1 group plants' gametes ready to fertilization (Figure 9a, b, c). During 25th stage Ψ_{pd} values for all groups -except Control- were -0.4 to -0.5 MPa. Seen in Figure 9d and 9e, WS_2 and WS_3 fertilized earlier than WS_0 and WS_1. And this sampling time was called late flowering.

In 27th stage while Ψ_{pd} values of control was about -0.2 MPa, in WS_1 and WS_2 these values were between -0.5 to -0.6 MPa. As for WS_3 severe to high water stress (< -0.6 MPa) occured. Therefore the growth of berries in WS_3 were slowed down after berry set. Berry set finished as it was seen in Figure 10f. In Figure 10a, shown that the egg cell get ready for double fertilization. Mullins et al. (1992) reported that the double fertilization is the process in grapevines in which two sperm nuclei from each pollen tube fertilize two cells in an ovary. It was shown in Figure 10e, one of the two sperm cells fertilizes the egg cell forming a diploid zygote. This was the point when fertilization actually occured. The other sperm cell fuses

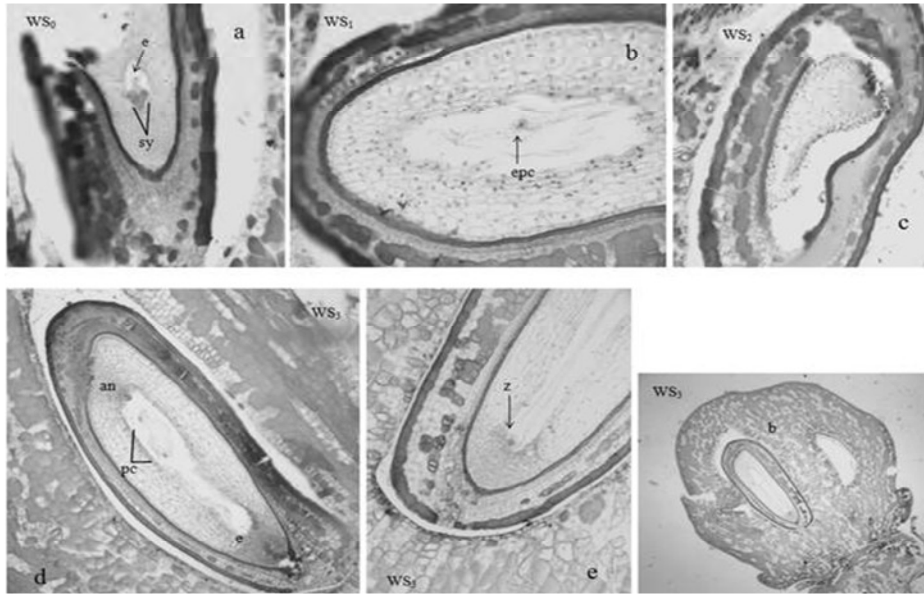

Figure 10. The double fertilization occured in 7th sampling time (27th E-L Stage). In WS_0 (a) (X20) fertilization was about to begin; double fertilization in WS_1 (b) (X20), WS_3 (d) (X10), WS_3 (e) (X20); nucellar cells seen in WS_2 (c) (X20); berry development started in WS_3 (f) (X4).
pc, Polar cells; *e*, egg cell; *an*, antipod cells; *sn*, synergids; *epc*, endosperm primer cell; *em*, embryo; *b*, berry.

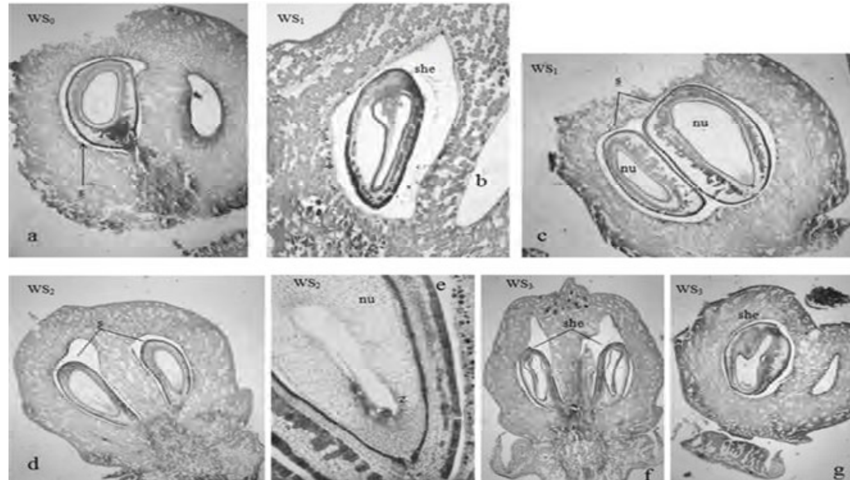

Figure 11. 8th sampling time, in which berry set (27th E-L Stage) was completed. But some malformations were seen in longitudinal sections. Normal seed growing seen in WS_0 (a) (X4), WS_1 (c) (X4) and WS_2 (d) (X4). Shrinking seeds were seen in WS_1 (b) (X10), WS_3 (f) (X4), WS_3 (g) (X4). Normal zygote development was seen in WS_2 (e) (X20). *z*, Zygote; *nu*, nucellar cells; *b*, berry; *s*, seed; *she*, shrunken seed.

with two haploid polar nuclei (Figure 10d) in the centre of the embryo sac. The resulting cell was triploid (Figure 10b). In Figure 10c, nucellar cells shrinked and went through the left side of ovule, concequently berry set occurred (Figure 10f).

After fertilization the pattern of cell division follows that of the Geum variation of the Asterad type. The pattern of endosperm formation in the grapevine is classified as a helobial (Maheshwari, 1950; Prat, 1971; Mullins et al., 1999) (Figure 11a, c, d, e). During this period (27th E-L Stage) while Ψ_{pd} values of WS_1 and WS_2 were between -0.4 and -0.6 MPa, in WS_3 Ψ_{pd} values decreased until -

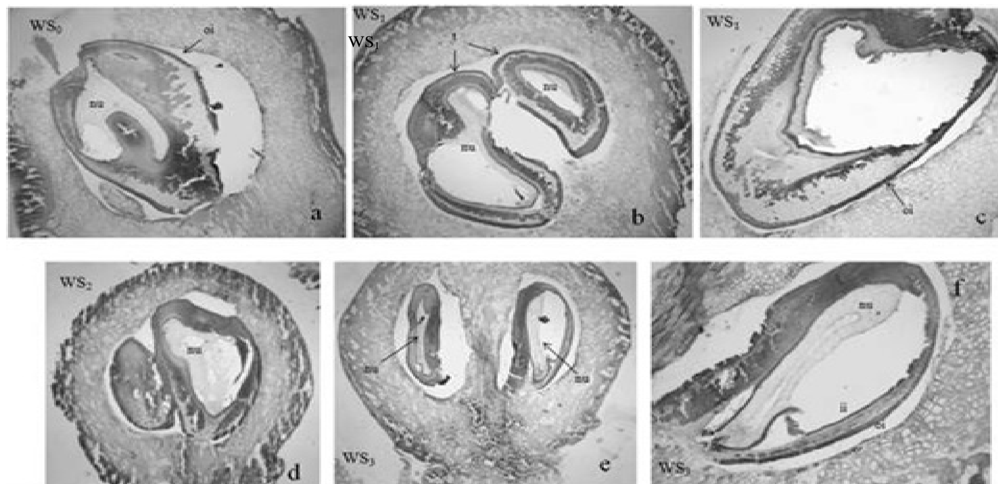

Figure 12. Sampling time 9 (29th stage of E-L). Normal seed development was seen in WS_0 (a) (X4) and in WS_1 (b) (X4). Some malformations occured in WS_1 (c) (X4), in WS_2 (d) (X4), in WS_3 (e) (X4), in WS_3 (f) (X10). *t*, Testa; *nu*, nucellar cells; *oi*, outer integument; *ii*, inner integument.

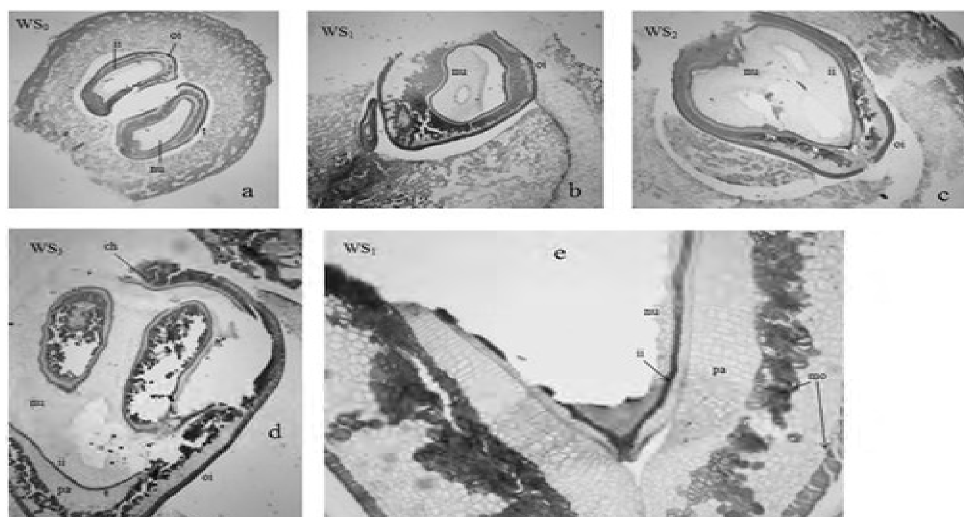

Figure 13 Sampling time 10, and 31st Stage according to the E-L Stage. Normal development of seed in WS_0 (a) (X4), in WS_1 (b) (X4), in WS_2 (c) (X4), in WS_3 (d) (X4). Layers of inner and outer integuments in micropylar side in WS_1 (e) (X4). *t*, Testa; *ii*, inner integument; *oi*, outer integument; *pa*, inner palisade layer; *mo*, middle and outer layers; *ch*, chalaza; *nu*, nucellus; *en*, endosperm.

0.78 MPa (severe to high water deficit). As it is shown in Figure 11b, f and g, structural defects of nucellar cells occurred in WS_1 and WS_2, especially in WS_3.. The primary endosperm nucleus divided and transverse wall was formed across the embryo sac, forming a small chalazal cell and a large micropylar cell (Figure 12a, b, d). Within the micropylar cell, the nucleus enters into free divisions; up to six divisions occur in the absence of wall formation. Within the chalazal cell every division is accompanied by wall formation (Figure 12c, e, f) (Mullins et al., 1992). After the measurements and samplings re-irrigation was started for all stress groups. In Figure 12c, thin nucellar cells were clearly seen and it was adhered to inner integument layer. In Figure 12f, nucellar cells shrunked and thus nucellus became narrow. Throughout this period Ψ_{pd} values in all stress groups increased suddenly because of re-irrigation (in WS_1 and WS_2 -0.3 and -0.4 MPa; in WS_3 -0.5 and -0.6 MPa). After fertilization there was a period of rapid cell division in the funiculus, raphe, chalaza and integuments. At the micropyle, the outer integument thickened and elongated to a form as the beak (Figure 13a to d). Early in development, the palisade layer consist of one layer of cells which then undergo cell division to form a double

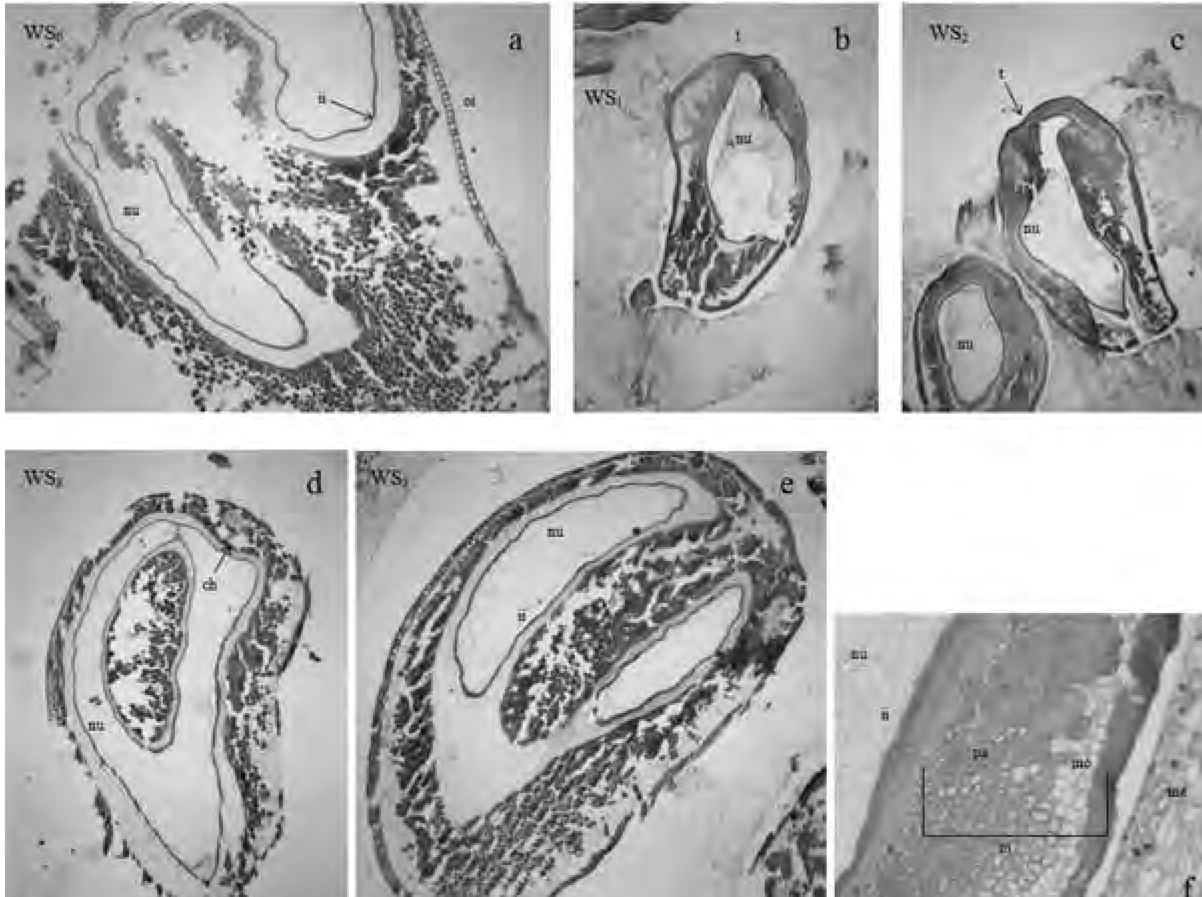

Figure 14. Last sampling time (11th), berries pea sized stage (31st E-L Stage). Development of seed and malformation in nucellus were seen in WS_0 (a) (X4), in WS_1 (b) (X4), in WS_2 (c) (X4), in WS_3 (d) (X4), in WS_3 (e) (X4), outer integument layer in (f) (X4). *t*, Testa; *ii*, inner integument; *oi*, outer integument; *pa*, inner palisade layer; *mo*, middle and outer layers; *ch*, chalaza; *nu*, nucellus; *me*, mezocarp.

layer (Walker et al., 1999). However, towards the chalaza the outer integument becomes folded and ridge is produced at the raphe on either side of which are two depressions or grooves known as fossettes (Mullins et al., 1992).

More also, 21 days after berry set the pincer-like growth of the outer integument squeezed the nucellus and endosperm into W-shape (Figure 14b). The testa comprised an outer integument of three layers and an inner integument that surrounded the nucellus (Figure 14a, c, d, e). The inner layer of the outer integument was a layer of palisade cells which overlied a layer of transfer cells in the inner integument (Figure 14f). The hardening of the grape seed is due to lignification of the inner layers of the outer integument (Walker et al., 1999).

Conclusion

To sum up; the polen viabilities and polen germination ratios were not affected by water stress. An early water stress caused poor berry set. WS_3 (severe to high water deficit) affected the phenologic stage and the berry set stage was long. In Merlot cv. which is sensitive to coulure (shattering) while Ψ_{pd} values were between 0 and -0.4MPa berry set was not affected much from 19th to 29th stage and there were not many differences (WS_0= 44.38%; WS_1= 38.93%).

Also from 21st to 29th stage while Ψ_{pd} values decreased to below -0.4MPa, there was about 45% decrease in berry set. - Malformations in seed development determined in WS_1, WS_2 and WS_3 when the Ψ_{pd} values decreased below -0.4MPa. As a result in Merlot cv. early water stress (between 19th to 29th stages) should be avoided especially when Ψ_{pd} values decrease below -0.4MPa.

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