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Review Article

Early flowering species - model plants for studies of ontogenesis *in vitro*

Aleksandra Lj. Mitrović[1*] Slavica Dmitrović[2] and Branka D. Živanović[1,3]

1 Institute for Multidisciplinary Research, University of Belgrade, Kneza Višeslava 1, 11030 Belgrade, Serbia

2 Institute for Biological Research "Siniša Stanković", University of Belgrade, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia

3 School of Land and Food, University of Tasmania, Private Bag 54, Hobart, Tasmania 7001, Australia

ABSTRACT: *Chenopodium rubrum* L. and *Chenopodium murale* L. are two annual weed species with different photoperiodic demands. The use of species from the same genus, with a similar type of morphogenesis but with different photoperiodic demands, is valuable for comparative studies of flowering. In addition, being classified as early flowering species, these two species represent model plants suitable for studies of ontogenesis *in vitro*. This review describes part of our results obtained on these two model plants under the guidance of Professor Ljubinka Ćulafić, starting with early development, such as somatic embryogenesis, followed by photoperiodic and hormonal regulation of flowering, to the photoperiodic control of different stages of ontogenesis.

Keywords: *Chenopodium rubrum* L., *Chenopodium murale* L., ontogenesis, flowering, *in vitro*, photoperiod.

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INTRODUCTION

With gratitude to Professor Ljubinka Ćulafić, this review is a brief summary of our work on model plants: *Chenopodium rubrum* L. and *Chenopodium murale* L.. It represents a contribution to the Chailakhyan" School of flowering (Chailakhyan 1937), comprising work of his students and followers in Moscow and Prague (Krekule & Seidlová 1976; Krekule 1997; Bavrina *et al.* 2002; Krekule & Ćulafić 2002).

We introduced the *in vitro* culture of intact plants into investigations of *C. rubrum* and *C. murale* photoperiodic and hormonal control of flowering (Živanović & Ćulafić 1992; Živanović *et al.* 1995; Mitrović *et al.* 2000a,b; Mitrović *et al.* 2003) and photoperiodic control of ontogenesis (Mitrović *et al.* 2002; Mitrović *et al.* 2007, Mitrović *et al.* 2010). In addition, a system for somatic embryogenesis was established (Milivojević *et al.* 2005) with the idea to obtain genetically uniform material for further research.

Early flowering plants. Plants able to be induced to flower at an early stage of development could be very useful for studies of flowering and consequently ontogenesis (Cumming 1967). Therefore in studies where large numbers of plants of successive generations, are required in a limited time and space, early flowering plants and *in vitro* culture are the right choices.

Many *Chenopodium* spp. (family Chenopodiaceae, genus *Chenopodium*) of different photoperiodic demands (long day-, short day- and day-neutral plants) belong to early flowering plants (Cumming 1967). Photoperiodic response within a species can differ greatly according to the latitude of its origin (Cumming 1967), demonstrating the importance of using seed lots from the selected population, i.e. chosen selection or ecotype.

C. rubrum (Fig. 1C) and *C. murale* (Fig. 1H) are weedy annuals, widely distributed in Europe, Asia and Northern America, and both are classified as early flowering plants (Cumming 1967). The use of species of the same genus (in our case genus *Chenopodium*) with a similar type of

*correspondence: mita@imsi.rs

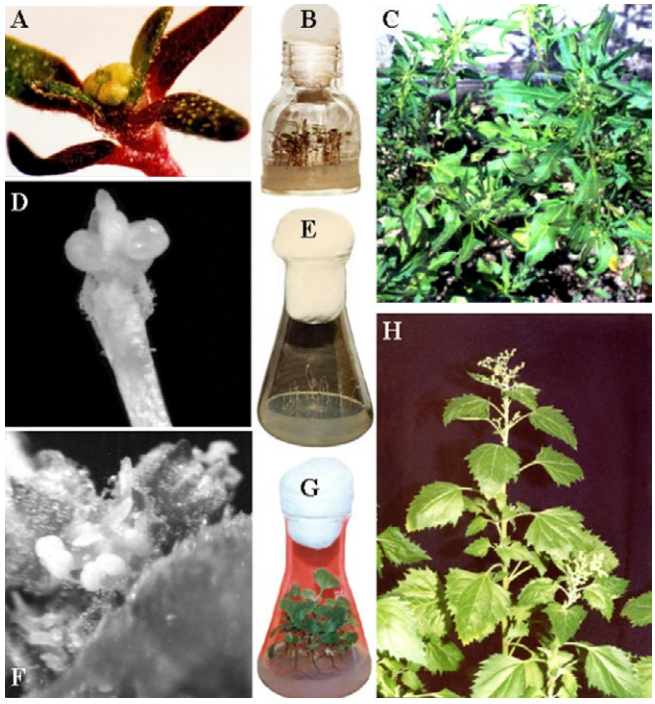

Figure 1. *Chenopodium rubrum* (A-E) – A, B) flowering plants on MS medium containing glucose (5%), C) flowering plants in a greenhouse, D, E) flowering in darkness on MS medium containing glucose (5%); *Chenopodium murale* (F-H) – F, G) flowering plants on MS medium containing glucose (5%), H) flowering plants in a greenhouse.

morphogenesis but with different photoperiodic demands (short-day plant *C. rubrum* and long-day plant *C. murale*) could be valuable for comparative studies of flowering and its regulation (Pavlová *et al.* 1989b).

Flowering *in vitro*. The transition from the vegetative to reproductive phase of development is controlled by genetic (autonomous) and ecological (photoperiod and temperature) factors. Autonomous control is age dependent, while induced or photoperiodic control (alone or in combination with temperature) modifies genetically-determined flowering.

One of the approaches to the analysis of hormonal signals in flowering has been exogenous application of phytohormones, where culture of intact plants *in vitro* has an advantage over a greenhouse (Scorza 1982). Auxin and cytokinin effects on flowering have been explained mainly through their regulation of apical dominance (Krekule 1979). Podolny *et al.* (1991) suggested a role of for auxins in both the perception and realization of flowering, while Bernier *et al.* (1993) considered cytokinins as one of the components of the floral stimulus. Promotion as well as inhibition of flowering was reported in various plants as a result of application of both auxins and cytokinins, with neither one being able to compensate for unsuitable photoperiodic conditions for flowering. Gibberellins are the only group of phytohormones able to substitute for photoperiodic flowering induction, but mainly in long-day plants. Chailakhyan (1958) suggested gibberellins as a component of the flowering hormone "florigen", while his coworker Seidlová (1985) showed that GA_3 has a promoting effect on *C. rubrum* flowering under the threshold induction. We suggested that GA_3, by showing a cumulative stimulatory effect with glucose on *C. murale* flowering, affects flower development rather than flower initiation (Mitrović *et al.* 2000a).

The interaction of saccharides and light to achieve reliable flower induction and development in a number of species *in vitro* has been previously reported by Scorza (1982). Sucrose and glucose in the media showed no difference concerning their effect on *in vitro* flowering in both *C. rubrum* and *C. murale* (Živanović *et al.* 1995). Within a series of experiments conducted to define the effect of sugars, as products of photosynthesis, on flowering, we investigated *C. rubrum* and *C. murale* flowering in darkness and flowering of SANDOZ 9789-treated "white" plants (Živanović *et al.* 1995; Mitrović *et al.* 2000b).

For a number of short-day-, but also long-day plants, flowering in darkness has been reported, mainly in the presence of sugars in the media (Bernier *et al.* 1981). The perception and transduction of the light signal involved in the photoperiodic flowering response is performed by a series of photosensitive systems (photoreceptors), including different forms of phytochromes (Sineshchekov 1999) and cryptochromes (Khurana & Poff 1999). Chailakhyan *et al.* (1987) suggested the use of SANDOZ 9789-treated "white" plants to exclude the effect of sugars, as products of photosynthesis, on flowering. SANDOZ 9789 treatment results in plants devoid of carotenoids and chlorophyll, without interference with the phytochrome system (Jaben & Deitzer 1979) or cryptochromes (Bavrina *et al.* 2002). Performing the comparative study of green and "white" *C. rubrum* plants under various culturing conditions, Bavrina *et al.* (2002) suggested the interaction of different photosystems in the control of flowering - the induction being dependent on phytochromes and cryptochromes, whereas the accomplishment of flowering depends on chlorophylls and carotenoids.

Chenopodium rubrum. *Chenopodium rubrum* L. Sel. 184 is a qualitative short-day plant, with a defined critical night length of 8 h (Tsuchia & Ishiguri 1981), sensitive to photoperiodic stimulus for flowering as early as at the cotyledonary stage (Seidlová & Opatrná 1978), when six adequate photoperiodic cycles are sufficient for photoperiodic flower induction both in the greenhouse (Seidlová & Opatrná 1978) and *in vitro* (Živanović & Ćulafić 1992).

Uniform germination (Cumming 1963), five days after the start of imbibition, resulted in seedlings with

fully-developed and opened cotyledons (Živanović & Ćulafić 1992). A photoperiod of 14 h/10 h was chosen as inductive (Živanović & Ćulafić 1992). Six cycles of inductive 14 h/10 h photoperiod followed by 9 cycles of non-inductive 18 h/6 h photoperiod resulted in flowering *in vitro* (Živanović *et al*. 1995), i.e. *C. rubrum* plants under suitable photoperiodic conditions *in vitro* flowered in 15 d (Fig. 1A, B). Even on sugar-free media flowering was 81%, while the addition of glucose (3-7%) resulted in 100% flowering (Živanović *et al*. 1995), confirming the participation of saccharides in the control of flowering (Corbesier *et al*. 1998). The addition of benzylaminopurine (BAP) (0.1-10 mg dm^{-3}) and indole-3-acetic acid (IAA) (0.1-10 mg dm^{-3}) inhibited, while gibberellic acid (GA_3) stimulated both growth and flowering (Živanović *et al*. 1995), showing a close relationship between growth of vegetative organs and photoperiodic flowering induction. Under non-inductive photoperiodic conditions (15 d of 18 h/6 h) neither sugars nor phytohormones in the media were able to compensate for *C. rubrum* requirements for flowering (Živanović *et al*. 1995).

For the qualitative short-day plant, *C. rubrum*, continuous darkness (CD) represents photoperiod induction for flowering. *C. rubrum* exposed to 15 d of CD flowered up to 80% (Mitrović *et al*. 2003). On sugar-free media there was no flowering, as sugars in the media are necessary for flowering in darkness to compensate for the lack of photosynthesis (Bernier *et al*. 1981), but as expected, glucose (2-10%) and GA_3 (1-5 mg dm^{-3}) stimulated flowering (Fig. 1D, E) in darkness (Mitrović *et al*. 2003).

SANDOZ 9789-treated *C. rubrum* "white" plants showed growth inhibition compared with green plants on sugar-free media. Glucose (5%) restored flowering up to 30%, while with GA_3 (0.10 – 10 mg dm^{-3}) it was up to 100% (Živanović *et al*. 1995). SANDOZ 9789 causes the destruction of chloroplasts, where photosynthesis as well as gibberellin synthesis take place (Rappaport & Adams 1979). Thus, media supplemented with sugar and GA_3 compensate for the lack of both sugars as products of photosynthesis and gibberellins, in "white" plants. Similarly to their effect on green plants, IAA and BAP (0.10 – 10 mg dm^{-3}) inhibited the growth and flowering of "white" plants (Fig. 2A, B, C) (Živanović *et al*. 1995).

Chenopodium murale. *Chenopodium murale* L. Sel. 197 is a facultative long-day weedy annual (Cumming 1967). It is sensitive to photoperiodic flowering induction as early as at the phase of the 1st pair of leaves (Fig. 1 F). Its photoperiodic sensitivity shows oscillatory changes with aging, expressed by the number of leaves (Pavlová *et al*. 1989a; Mitrović *et al*. 2000a). As a facultative long-day plant, continuous light (CL) was selected (10 d of CL) as a photoperiod induction for flowering, while short days (8 h/16 h light/dark) were used as a non-inductive photoperiod – (Pavlová *et al*. 1989b). As the plant is not photoperiod sensitive until the development of the 1st pair of leaves, and as its photoperiodic sensitivity shows oscillatory changes, plants were grown under a 8 h/16 h photoperiod until 1st, 2nd or 3rd pair of leaves were developed, then exposed to photoperiodic flower induction (10 d of CL) and returned to a non-inductive photoperiod for realization of flowering (Mitrović *et al*. 2000a).

The presence of sugar in the media was necessary for *C. murale* flowering (Mitrović *et al*. 2000a). Glucose (5%) in the medium resulted in 17% of flowering when induced at the 1st pair of leaves (Fig. 1F, G). With aging, *C. murale* shows oscillatory changes of photoperiodic sensitivity (Pavlová *et al*. 1989b; Mitrović *et al*. 2000a), but also loosening of photoperiodic control (Bernier *et al*. 1981; Mitrović *et al*. 2003). Sensitivity to the photoperiod at the stage of the 2nd pair of leaves was reduced (Pavlová *et al*. 1989b; Mitrović *et al*. 2000a); at the 3rd pair of leaves it was restored and increased, while at the 4th pair of leaves loosening of photoperiodic flowering control was registered (Mitrović *et al*. 2003).

BAP (0.1-5 mg dm^{-3}) and IAA (0.1-5 mg dm^{-3}) inhibited, while GA_3 (0.1-10 mg dm^{-3}) stimulated both growth and flowering. GA_3 alone or in combination with glucose in the medium stimulated flowering up to 67% in plants induced to flower at the stage of the 3rd pair of leaves (Mitrović *et al*. 2000a,b).

Under non-inductive photoperiodic conditions (45 short 8 h/16 h days), neither sugars nor phytohormones in the media were able to compensate for *C. rubrum* requirements for flowering (Mitrović et al. 2000a).

For a long-day plant such as *C. murale*, continuous darkness is a non-inductive photoperiod, although we showed that exposure to 10 d of CD at the stage of the 4th pair of leaves, it flowered up to 65% with the addition of glucose (5%) and GA_3 (5 mg dm^{-3}) in the medium (Mitrović *et al*. 2003). From this, *C. murale* can be added to the list of long-day plants able to flower in darkness: *Rudbeckia bicolor* (Chailakhyan 1988), *Beta vulgaris* (Fife & Price 1953), *Triticum aestivum* (Sugino 1957). In relation to this, the same results (60% flowering) were obtained in *C. murale* plants induced to flower at the stage of the 4th pair of leaves by 10 d of CL with the addition of glucose (5%) and GA_3 (1 mg dm^{-3}) (Mitrović *et al*. 2003). *C. murale* loses its requirement for a specific photoperiod for flowering with aging, so exposure to CD cancels the photoperiodic control of flowering and flowering occurs under autonomous mechanisms (Mitrović *et al*. 2003).

SANDOZ 9789-treated *C. murale* "white" plants did not survive on a sugar-free medium. The addition of glucose (5%) to the media enabled of "white" plants to survive with significant inhibition of growth compared with green plants (Mitrović *et al*. 2000b). Similarly to *C. rubrum*, IAA and BAP (0.10 – 5 mg dm^{-3}) inhibited,

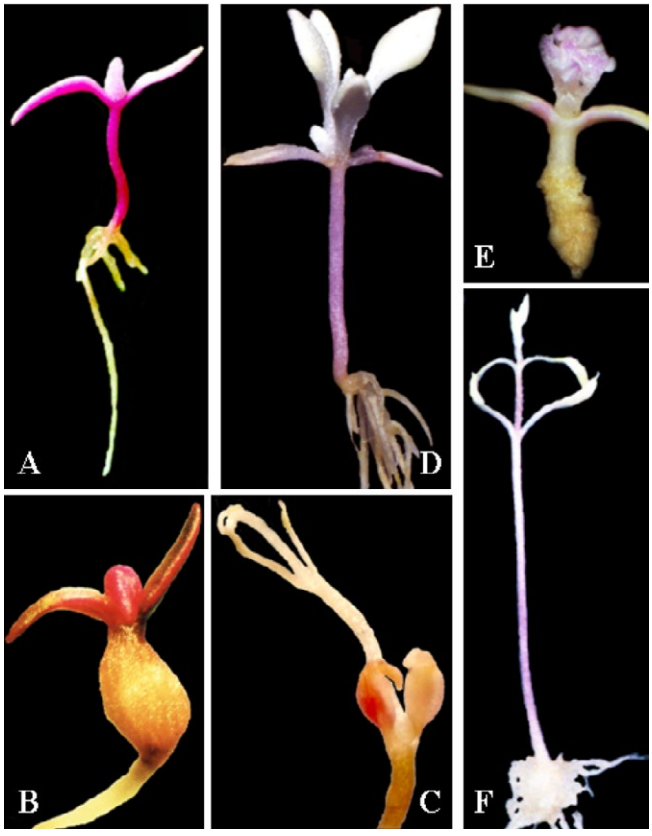

Figure 2. *Chenopodium rubrum* (A-C) - A) SANDOZ 9789-treated "white" plant, B – "white" plant on medium containing BAP (5 mg dm^{-3}), C) "white" plant on medium containing GA_3 (5 mg dm^{-3}); *Chenopodium murale* (D-F) - D) SANDOZ 9789-treated "white" plant, E) "white" plant on medium containing BAP (5 mg dm^{-3}), F) "white" plant on medium containing GA_3 (5 mg dm^{-3}).

while GA_3 (10 mg dm^{-3}) stimulated the growth and flowering of "white" plants (Fig. 2 D, E, F).

Photoperiodic control of *Chenopodium rubrum* ontogenesis *in vitro*. Altering day-length in plants such as *C. rubrum* sel. 184 with a well-defined critical night length and sensitivity to photoperiod (Tsuchiya & Ishiguri 1981; Seidlová & Opatrná 1978) is a valuable source of information on the regulation of plant development in accordance with photoperiod. Seedlings with fully-developed cotyledons were exposed to different photoperiodic treatments for 10 weeks: a 8 h/16 h, 14 h/10 h, 16 h/8 h photoperiod, 6 d of 8 h/16 h followed by 9 weeks of 16 h/8 h photoperiod, or 6 days of a 14 h/10 h followed by 9 weeks of a 16 h/8 h photoperiod. With the increase of day-length from 0 h to 24 h, plant height was increased, flowering was delayed, seed development occurred earlier, and plants produced more seeds (Mitrović *et al.* 2007). Key processes in *C. rubrum* development, growth pattern to the end of ontogenesis, flowering and seed development, are all determined by the photoperiod the seedlings experience during the early and specific period of their life cycle – the first 6 d after cotyledon opening, i.e. a period in their life cycle when they are sensitive to photoperiodic flower induction (Mitrović *et al.* 2007; Cook 1975).

However, the effect of photoperiod on *C. rubrum* plants does not end there. The photoperiod experienced during ontogenesis of mother plants also affected the vegetative and reproductive development of their offspring (Mitrović *et al.* 2010). Environmental effects on morphological and physiological characteristics of the resultant seeds (offspring), which took place during the development of mother plants, are called maternal environmental effects (Gutterman & Evenari 1972). Maternal environmental effects could be elicited by different environmental factors, their expression depending on the offspring environment and they may persist for several generations (Amzallag 1999; Galloway 2005). Therefore, we showed that the maternal effect of the photoperiod extends through the whole life cycle of *C. rubrum* offspring. On the basis of correlation analysis of relative intensities of seed protein bands obtained after SDS-PAGE electrophoresis and photoperiods that maternal plants were exposed to during their onthogenesis, we assumed that mother plants transfer to their seeds a "protein message" on the day-lengths they experienced during their life cycle (Mitrović *et al.* 2010). The photoperiod during flowering induction of mother plants has a key influence on seed germination and the growth of offspring, while offspring flowering and seed maturation is determined by the photoperiod experienced by their mothers during induction and evocation of flowering. In addition, maternal effects of photoperiod in *C. rubrum* persist to the second generation, as maternal photoperiod also affected offspring seed size and number (Mitrović *et al.* 2010). Natural *C. rubrum* flowering induction is associated with minimizing seed weight and maximizing seed number, favoring physiological mechanisms that work under suboptimal photoperiods, maximizing the probability to survive (Cook 1975).

Somatic embryogenesis. Somatic embryogenesis is defined as a process by which haploid or diploid somatic cells pass through characteristic embryonic stages without fusion of gametes, forming a complete plant (Jimenez 2005). Successful induction of somatic embryogenesis usually has been performed from young plant tissues due to their high regenerative potential (Al-Khayri 1997). Culture medium supplemented with auxins combined with cytokinins, in many plant species, provides for successful somatic embryogenesis (Ammirato 1983; Van Staden *et al.* 2008). Sometimes implementation of two or more regulators is necessary for induction and development of somatic embryos (Tetu *et al.* 1987; Zdravković-Korać & Nešković 1998; Xiao & Branchard 1993; Sasaki *et al.* 1994).

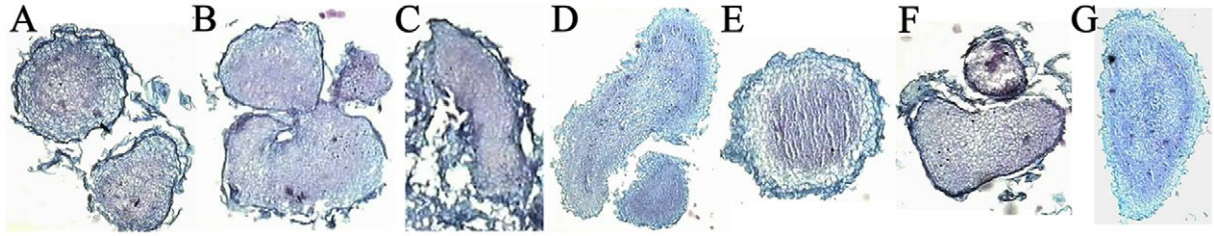

Figure 3. Histological sections of *Chenopodium rubrum* (A-D) and *C. murale* (E-G) somatic embryos in various stages of development: A) globular stage, B) heart stage, C) torpedo stage in scattered callus tissue, D) globular and older stages, E) globular stage, F) globular and heart stages, G) torpedo stage.

Michalczuk *et al.* (1992) and Pasternak *et al.* (2002) showed that 2,4-dichlorophenoxyacetic acid (2,4-D), the most used auxin for this purpose, in the culture medium increased the endogenous auxin content in the explants, which is crucial for somatic embryo formation (Fischer & Neuhaus 1996). Moreover, it has been shown that increased sucrose concentration in the culture medium leads to the initiation of somatic embryogenic culture in many plant species (Ćulafić *et al.* 1987; Finer 1987; Nešković *et al.* 1987; Lou & Kako 1994).

The kind of explants and optimum culture conditions to establish an efficient system for *in vitro* plant regeneration of *C. rubrum* and *C. murale* by somatic embryogenesis have been defined. In both species, somatic embryogenesis was induced on Murashige and Skoog (MS) medium supplemented with sucrose (3%), agar (0.7%) and 1-10 μM 2,4-D, while release of embryos from calli was performed on liquid culture medium with an increased sucrose content (6%). Calli formation was observed on the basal medium in *C. rubrum* and *C. murale*, but they were non-embryogenic. Somatic embryogenesis in both species was obtained using only one growth regulator (2,4-D) (Milivojević *et al.* 2005).

Chenopodium rubrum. With an increase of 2,4-D (from 1 to 10 μM) in MS media, a reduced number of somatic embryos was formed. Root explants showed the highest embryogenic capacity (Fig. 3) (Milivojević *et al.* 2005).

Chenopodium murale. Compared with *C. rubrum*, in *C. murale* the number of somatic embryos per explant increased with the increase of 2,4-D (from 1 to 10 μM) in MS media, in all explant types (hypocotyl, basal and apical part of the cotyledon, basal and apical part of the leaf and root). Basal parts of cotyledons showed the highest embryogenic capacity (Fig. 3) (Milivojević *et al.* 2005). *C. murale* showed a higher capacity for embryogenic calli production than *C. rubrum*.

CONCLUSION

Somatic embryogenesis, as an efficient system for *in vitro* plant regeneration of *C. rubrum* and C. *murale* was established. Exogenous application of saccharides, phytohormones and herbicide SANDOZ 9789 exhibited similar results on flowering and related growth in both species, regardless of their different photoperiodic demands. Even under an extreme photoperiod (continuous darkness), both species showed the ability to flower. We demonstrated that the photoperiod experienced early during the *C. rubrum* life cycle determines the whole pattern of its ontogenesis. We showed that the maternal effect of photoperiod in *C. rubrum* extends through the whole life cycle of its offspring and persists to the second generation, with the suggested mechanism being through changes in seed protein patterns.

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REZIME

Rano cvetajuće vrste – model biljke za istraživanja ontogeneze *in vitro*

Aleksandra Mitrović, Slavica Dmitrović, Branka D. Živanović

Chenopodium rubrum L. i *Chenopodium murale* L. su jednogodišnje korovske biljke sa različitim fotoperiodskim zahtevima. Mogućnost korišćenja vrsta iz istog roda, sličnog tipa morfogeneze, ali sa različitim fotoperiodskim zahtevima, je od neprocenjivog značaja za komparativna istraživanja cvetanja. Osim toga, klasifikovane kao rano cvetajuće vrste, ove dve vrste predstavljaju i model biljke prikladne za izučavanje ontogeneze *in vitro*. Ovaj revijski rad predstavlja deo naših rezultata dobijenih na ovim model biljkama, a pod budnim okom naše drage Profesorke Ljubinke Ćulafić, počev od somatske embriogeneze, preko fotoperiodske i hormonalne regulacije cvetanja, do fotoperiodske kontrole različitih faza ontogeneze.

Ključne reči: *Chenopodium rubrum* L., *Chenopodium murale* L., ontogeneza, cvetanje, *in vitro*, fotoperiod.