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Preservation of sweet chestnut genetic resources (*Castanea sativa* Mill.) against attack by chestnut gall wasp (*Dryocosmus kuriphilus* Yasumatsu, 1951)

Rebecca VOLLMEIER[1], Gregor OSTERC[1], Zlata LUTHAR[1*]

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ABSTRACT

European sweet chestnut (*Castanea sativa* Mill.) is one of the most important wood species due to its environmental and economic role in many agro-forestry systems. Chestnut gall wasp (*Dryocosmus kuriphilus* Yasumatsu, 1951) is currently the most dangerous pest of sweet chestnut, including in Slovenia. Attack on vegetative buds (in which the eggs are deposited and on which galls are subsequently formed) disturbs the growth of shoots and reduces the yield. In the event of a strong attack, the tree can weaken and decay, which is already noticeable on the ground in Slovenia, especially in terms of the monitored genetic resources of the chestnut tree. Following Japanese experience, European countries are increasingly choosing biological control of chestnut gall wasp with the torymid wasp (*Torymus sinensis* Kamijo, 1982). Micropropagation is a way of ensuring effective preservation and reproduction while optimizing all phases of work. In the micropropagation of Slovenian sweet chestnut genetic resources, problems arise in the rooting phase.

Key words: sweet chestnut; *Castanea sativa;* chestnut gall wasp; *Dryocosmus kuriphilus;* torymid; *Torymus sinensis;* micropropagation; breeding

IZVLEČEK

OHRANITEV GENSKIH VIROV EVROPSKEGA PRAVEGA KOSTANJA (*Castanea sativa* Mill.) PRED NAPADOM KOSTANJEVE ŠIŠKARICE (*Dryocosmus kuriphilus* Yasumatsu, 1951)

Evropski pravi kostanj (*Castanea sativa* Mill.) je ena izmed najpomembnejših lesnatih vrst zaradi svoje okoljske in gospodarske vloge v mnogih kmetijsko-gozdnih sistemih. Trenutno je kostanjeva šiškarica (*Dryocosmus kuriphilus* Yasumatsu, 1951) tudi v Sloveniji najpomembnejši škodljivec pravega kostanja. Z napadom vegetativnih brstov, kamor odlaga jajčeca in posledično povzroča tvorbo šišk, moti rast poganjkov in zmanjšuje pridelek. Ob močnem napadu lahko drevo zelo oslabi in propade, kar je na terenu v Sloveniji že opazen pojav, predvsem pri spremljanih genskih virih kostanja. Po vzoru japonskih izkušenj se tudi evropske države vse pogosteje odločajo za biotično varstvo s parazitoidno oso *Torymus sinensis* Kamijo, 1982. Mikropropagacija je način, ki zagotavlja učinkovito ohranjanje in razmnoževanje ob optimizaciji vseh faz dela. Pri mikropropagaciji slovenskih genskih virov se pojavljajo težave v fazi koreninjenja.

Ključne besede: evropski pravi kostanj; *Castanea sativa;* kostanjeva šiškrica; *Dryocosmus kuriphilus;* parazitoid; *Torymus sinensis;* mikropropagacija; žlahtnjenje

1 INTRODUCTION

Sweet chestnut is a woody species that plays an important role in the world because of its wide functional value and because of its economic and environmental importance. Asian species, the Chinese chestnut, *Castanea mollissima* Blume, the Japanese chestnut, *C. crenata* Sieb. & Zucc. and another Chines species, *C. henryi* (Skan.) Rehd. & Wils., as well as the European chestnut , sweet chestnut, *C. sativa* (Mill.), have been a basic food for survival of the population for centuries in many parts of Asia, Southern Europe and most of the countries bordering the Mediterranean (Bounous, 2005).

The Romans spread European sweet chestnut (*C. sativa* Mill.) throughout the European continent mainly to produce wooden barrels for storing wine. During this period, chestnut as a source of food was not the main reason for its spread across Europe. Growing sweet chestnut as food for sustenance was mainly developed after the Roman period, in connection with the socio-

[1] Department of Agronomy, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, *corresponding author: zlata.luthar@bf.uni-lj.si

economic system of the Middle Ages. The ancient Greeks were important growers of sweet chestnut for wood and fruit, although they never grew it on a large scale (Metaxas, 2013). In Europe, the Greeks were among the first to use the fruit and introduce the sweet chestnut to other cultures, from Asia Minor to Southern Europe and North Africa. Chestnut species and cultivars are traditionally grown today in China, Korea, Japan and the Mediterranean (Litz, 2005).

Except for timber extraction and tannin production, sweet chestnut fruit and honey are the only market-relevant crops within the family of Fagaceae in the temperate zone. Despite increasing demand, global chestnut production has been gradually decreasing over the last century, in particular due to fungal diseases and pests that have not only destroyed the chestnut population throughout its distribution but have also limited the creation of new growing chestnut areas (Litz, 2005; Metaxas, 2013).

There are three main areas of chestnut plantations around the world: (1) in Asia the most important area is in China, where the species *C. mollissima* and *C. henryi* grow in natural conditions, as well as in plantations, and in Japan, where the species *Castanea crenata* is widespread; (2) Europe is the second main area, in which the species *C. sativa* is predominant; (3) in North America, the American chestnut, *C. dentata* (Marsh.) Borkh. was widespread in nature, but is nowadays replaced by hybrids that are resistant to chestnut blight and ink disease (Pereira-Lorenzo and Ramos-Cabrer, 2007).

European sweet chestnut (*C. sativa*) is widespread in forests and artificial plantations across all Mediterranean countries and elsewhere. It extends from Spain and Portugal to the Caucasus, across Turkey, Greece, the Balkans, the former Soviet Union and the southern part of Great Britain. In North Africa, it is found in small areas of Morocco, Algeria and Tunisia. The main producers in Europe are Italy, Turkey, Portugal, Spain, France and Greece. Chestnuts are no longer a matter of survival in Europe but they still play an important role in diet, the timber industry and landscape strategies in many agri-economic systems. Over the past thirty years, the chestnut ecosystem has increased its ecological and landscape significance mainly through the planting of varieties resistant to fungal diseases and it has thus become a fundamental resource for the sustainable development of mountain areas (Bounous, 2005; Bounous, 2014).

Due to the rapid expansion of the chestnut gall wasp with planting material from the original areas of China to almost all of the world's growing areas, including Europe, and the rapid collapse of trees, new plant breeding programs have begun to appear, in order to obtain genotypes resistant to this very dangerous pest.

2 CHESTNUT GALL WASP: DESCRIPTION AND ITS SUPERVISION

The great dispersal of and economic damage by the chestnut gall wasp (*Dryocosmus kuriphilus* Yasumatsu, 1951) is a consequence of trade in or transfer of attacked planting material. Mathematical models have assessed the natural spread of the chestnut gall wasp to be at a speed of 8 km per year, with a variation ranging from 3 to 12 km $year^{-1}$, which reflects the rate of natural spread of this species to other parts of Europe (EFSA, 2010; Gibbs et al., 2011).

By attacking the vegetative buds, through the development of the wasps the chestnut gall wasp disrupts the growth of shoots, the flow of assimilates and reduces the crop. In both commercial plantations and natural stands, the crop can be reduced from 50 – 70 %, depending on the severity of the attack. Severe damage can cause complete crop decline and the death of the tree. Chestnut gall wasp is still the worst insect pest of chestnut trees globally (*Dryocosmus kuriphilus*, 2005). Some researchers argue that severe and repeated injuries over several years gradually lead to a decline in vitality and often lead to the destruction of trees (Bosio et al., 2010). This was confirmed by our field visits carried out from 2003 for obtaining material for the design of *in vitro* cultures. At that time, we did not notice any damage from the chestnut gall wasp (Osterc et al., 2005). We sampled the material again in 2015 and 2016, and found that almost all the genetic resources that had been previously marked around Slovenia had been severely attacked by chestnut gall wasp and were rapidly decaying. In 2015, complete collapse was observed in the genotype from the surroundings of Pedrovo (Primorska), which was also supported by the age of the trees.

2.1 Physical and phytosanitary measures to suppress the chestnut gall wasp

Damage in small chestnut stands can be reduced by pruning and destroying damaged shoots but commercial growers do not make use of this due to the cost and uncontrollability of work. Insecticides can be effective against adult female and young larvae but with negative side effects on the environment (*Dryocosmus kuriphilus*, 2005; Knapič et al., 2010). Most insecticides do not provide good control over the chestnut gall wasp. Systemic insecticides have an advantage over contact ones, since most of the developmental phases (eggs, larvae, bugs and part of the adult wasps) are protected in the galls. Several experiments have been carried out in the past in which very toxic insecticides were used. In China, good efficacy was achieved by injecting methanmidophos and ometoate into tree trunks and by spraying with dichlorvos, methylparation and methamidophos (Bosio et al., 2010).

In Italy, the Piedmont Region Plant Health Service, in collaboration with local research centres for fruits and vegetables, studied the effectiveness of some insecticides permitted in other fruit species but not chestnuts. Treatments were carried out in various phenological phases of young plants and development stages of the pests, in both nurseries and plantations. Treatments that were carried out at the time of the appearance of adult gall wasps leaving the kaolin galls, with a mineral powder that acts as a physical barrier or with lambda-cyhalothrin, alpha-cypermethrin and ethylchlorpyrofos with the addition of mineral oil, contributed to minor injuries to the plant tissue but increased the mortality of adult females and reduced the appearance of eggs in the buds. These results were obtained from young plants grown in pots. However, it took five to six treatments to protect plants during the flying season of the gall wasps. This means that it would be very expensive on larger trees, and an unacceptable technique for the environment. In addition, the risk of killing pollinators and toxic residues in honey need to be considered, since the first treatments may also overlap with the time of chestnut flowering. It is thus obvious that chemical control would pose more risk than benefit. In the Piedmont region, therefore, they decided to follow the Japanese example and selected biological control (Bosio et al., 2010).

Strict monitoring of the transport of infected plant material can significantly reduce the proliferation of gall wasps over a long-distance and to new areas across Europe. There are currently very limited options for managing existing pest populations and reducing the extent of their impact. Since the larvae and pupas of the pest are protected within galls, conventional chemical protection is very ineffective. The development of resistant varieties of *Castanea* sp. is a solution for new chestnut plantations but does not solve the spread of pests from existing attacked areas.

After the Second World War, Japanese fruit growers chose varieties of chestnut with some resistance, but the pest developed a new strain to overcome this resistance (*Dryocosmus kuriphilus*, 2005).

In some parts of China, where the pest is naturally present, populations of the chestnut gall wasp survive in small numbers but do not cause economic damage, probably due to natural enemies, but there has been little publicity and there is little knowledge of alternative sources of pest mortality in these areas (Gibbs et al., 2011).

2.2 A biological measure of suppression of the chestnut gall wasp

In its natural area of distribution in China, the chestnut gall wasp is controlled by natural enemies, especially indigenous Hymenoptera parasitoids. Many new parasitoids parasitizing the chestnut gall wasp have recently been described in China, Japan and Korea; e.g., *Torymus sinensis* Kamijo, 1982, *Torymus beneficus* Yasumatsu & Kamijo, 1979, *Megastigmus maculipennis* Yasumatsu & Kamijo, 1979, *Megastigmus nipponicus* Yasumatsu & Kamijo, 1979 (Chalcidoidea, Torymidae), *Ormyrus flavitibialis* Yasumatsu & Kamijo, 1979 (Ormyridae) and others. Some of these parasites have proved to be very effective (Yasumatsu and Kamijo, 1979; *Dryocosmus kuriphilus*, 2005; Kos and Trdan, 2010).

The parasitoid wasp *Torymus sinensis* (Kamijo) has been released as a biological control agent for controlling gall wasp populations in North America, Japan and Europe. The parasitoid seeks galls of chestnut gall wasp in early spring using visual and olfactory organs and hatches its eggs in a barnacle containing developing larvae of the pest. The parasitoid larvae feed on the larvae of the chestnut gall wasp and remain inside the galls until they are fully grown and leave the barnacle next spring (Graziosi and Rieske, 2015).

T. sinensis is a natural species in China and Moriya et al. reported in 2003 that this is the only Chinese chestnut gall wasp parasitoid species that was previously known and is also host specific and phenologically well coincides with chestnut gall wasp.

In 1979 and 1981, 260 females of *T. sinensis* (from about 5000 galls of chestnut gall wasps imported from China) were released as a biocontrol method on Japanese chestnut trees at a research station in the province of Ibaraki. By 1989, the population of *T. sinensis* had increased 25-fold and it had become the most common group of local chestnut gall wasp parasitoids. Following this increase, the parasitoid *T. sinensis* successfully expanded into areas with a population of Japanese chestnut gall wasp and achieved effective biological control. The parasitoid wasp *T. sinensis* was also released in the US state of Georgia in the late 1970s, in response to widespread chestnut gall wasp infestation in North America The number of damaged shoots decreased, ensuring effective biological control (Gibbs et al., 2011).

However, some sources claim that the imported natural parasitoids in Japan and the United States, and most probably in Europe, will not ensure good control of the chestnut gall wasp, since they are not specific and do not coincide with the life cycle of the pest, as well as there being various environmental effects (*Dryocosmus kuriphilus*, 2005).

Preliminary studies on the spread of the *T. sinensis* parasitic wasp were firstly carried out in Europe in 2003 and 2004 in Italy, using imported parasitoid Japanese galls of *D. kuriphilus.* In these initial studies, the phenological discrepancy between the emergence of adult parasitoid *T. sinensis* and the development of the local chestnut gall wasp *D. kuriphilus* (due to temperature changes that had occurred during the transfer) was shown. Based on these results the parasitoid wasps could not be released into the open field. Behavioural experiments were also used, and they helped to improve later efforts in controlling adult females. In 2005, several chestnut galls of *D. kuriphilus* were imported from Japan. Their development was slowed down by artificial cooling. This enabled artificial adjustment of the appearance of imported adult parasitoid *T. sinensis* with populations of the chestnut gall wasps *D. kuriphilus,* and females of the parasitoid wasp *T. sinensis* were then released at three sites in Italy attacked by the chestnut gall wasp *D. kuriphilus*. Following successful adjustment of the *T. sinensis* parasitoid wasp at all three sites, a further cultivation program was set up to stimulate the release of the parasitoid *T. sinensis* to additional attacked areas in Italy (Gibbs et al., 2011).

However, it is still too early to assess the long-term effectiveness of biological control by the parasitoid wasp *T. sinensis* against Italian chestnut gall wasp populations of *D. kuriphilus*. Successful control would mean reducing chestnut damage to less than 30 % (Gibbs et al., 2011).

Further studies and confirmation of the effectiveness of the parasitoid wasp (*T. sinensis*) as a viable option for biological control of the chestnut gall wasp (*D. kuriphilus*) in Central Europe is indispensable. It has been suggested that more attention should be paid to determining: (i) the conditions under which the parasitoid wasp (*T. sinensis*) can attack the host chestnut gall wasp and (ii) the probability of the two genera crossing. Both issues are central to predicting the spread of the released parasitoid wasp (*T. sinensis*) and assessing the environmental risks associated with a more widespread release of this species into Europe (Gibbs et al., 2011).

2.2.1 Environmental risk assessment

A comprehensive assessment of environmental risk is based on the identification and evaluation of potential risks associated with the release, or planned dissemination, of a natural enemy and the development of a risk reduction plan. The last step before the introduction of a planned dissemination is to identify, evaluate and consider all negative and positive effects in terms of benefits, risks and costs.

The first question in the assessment of environmental risk relates to the origin and purpose of use of the biological controlling agent. It is necessary to evaluate the extent of the appearance of the parasitoid wasp (*T. sinensis*) and decide whether it can attack non-target species. Earlier studies have shown that wasps that cause galls on oak tree are attacked by similar parasitoids, so there is a general risk that the parasitoid wasp (*T. sinensis*) will pass over to autochthonous wasps that are related to chestnut gall wasps (*D. kuriphilus*), including an autochthonous species of the genus *Dryocosmus* that causes galls on oak trees. Information on cultivation and monitoring in China shows that the parasitoid wasp (*T. sinensis*) is very specific to chestnut gall wasp (*D. kuriphilus*). It should be noted that such an obvious monophagia is exceptional among many parasitoids that attack galls (Gibbs et al., 2011).

Two recent assessments of the parasitoid wasp (*T. sinensis*) as a candidate for biological control have shown many deficiencies in knowledge of the biology of this species. The key issue in relation to attacking non-target hosts is the latter's seasonal phenology and thus the potential for the parasitoid wasp (*T. sinensis*) to attack other hosts, in addition to chestnut gall wasp (*D. kuriphilus*). If *T. sinensis* could be confirmed as a specific parasitoid that does not attack non-target species, then it could be considered as a candidate for biological control of the chestnut gall wasp (*D. kuriphilus*) beyond the present scale. Conversely, if the parasitoid wasp (*T. sinensis*) has a wider host circle, it would be regarded as too risky to release, and unsuitable for biological control in other parts of Europe (Gibbs et al., 2011).

The possible crossing of a biological control organism with indigenous species is considered to be an environmental risk for non-target species and is, in general, a threat to autochthonous biodiversity. Theoretically, insects introduced as a biological control can cross with indigenous species. It is worth mentioning that the only such example so far reported involves *T. sinensis* and the Japanese autochthonous species *Torymus beneficus* Yasumatsu and Kamijo. It was suggested that there is a possibility of interbreeding, which was confirmed by the successful crossing of *T. sinensis* and *T. beneficus* under laboratory conditions and by gaining fertile hybrid females. Hybrids were also found in the field and molecular markers confirmed their hybrid origin (Gibbs et al., 2011).

A thoroughly designed risk assessment would confirm or deny the risk of expanding the introduction of *T. sinensis* that would outweigh the risks associated with the use of other control options (chemical control). More attention should be given to determining: (i) the conditions under which *T. sinensis* could attack alternative hosts and (ii) the probability of crossing with indigenous species of the genus *Torymus*. It is necessary to consider factors such as: the type of host, host behaviour in the given area, the location of the galls on host plant and phenology, since these factors can influence the outcome and reliability of host specificity tests (Gibbs et al., 2011). In general, current data suggest that the release of *T. sinensis* could have a wide range of potential impacts and it is therefore important to consider all possible impacts before further release of *T. sinensis* across Europe. The only alternative to the mentioned measures is breeding tolerant/resistant genotypes to reduce and weaken the pest population, which does not solve the current situation on the ground.

3 BREEDING OBJECTIVES AND LACK OF TOLERANT/RESISTANT VARIETIES

Most phytosanitary measures for suppression have not been shown in experimental studies effectively to eradicate or reduce the presence of chestnut gall wasp. There are also concerns about biological control, so an alternative is identifying resistance genes and the targeted breeding of resistant varieties or hybrids to replace damaged trees.

In Europe and Asia, the main breeding objectives have been focused on the selection of economically interesting genotypes from natural sites and obtaining hybrids with added genes for tolerance or resistance to severe diseases and pests, in order to improve and obtain resistant varieties (Bounous, 2014). In America, Australia and New Zealand, efforts are directed towards acquiring new varieties with the desired tolerance/resistance properties, or a selection of elite genotypes among the best European and Asian varieties, with wide adaptation to local pedoclimatic conditions. Other breeding goals to improve the properties of wood and fruit have recently been of secondary importance (Van Fleet, 2014). For the rapid replacement of decayed trees and cultivation in plantations, many studies have focused on successful vegetative propagation of resistant varieties with the best compatible properties of the rootstock and the graft, which has a significant impact on the survival and quality of the seedling. It is well established that *C. crenata* and *C. dentata* are more easily propagated than *C. sativa* (Galic et al., 2014). *C. crenata* is therefore often included as one of the parents in interbreeding with *C. sativa* to improve rooting. However, there is a problem of compatibility between the rootstock and the graft. It has been found that there is better compatibility between grafted scions that are hybrids of the same parents as the rootstock (Bounous, 2005).

Breeders have recently used molecular markers to improve approaches to detecting resistant genes against major fungal pathogens, such as chestnut blight (*Cryphonectria parasitica* (Murr.) Barr.) and ink disease caused by *Phytophthora cambivora* (Petri) Buis. and *P. cinnamomi* (Rands.). Genes for resistance to ink disease have been found in *C. crenata* and *C. mollissima*. Japanese-European hybrids *C. crenata* x *C. sativa* obtained in France: 'Marsol', 'Maraval', 'Ferosacre', 'Marigoule', 'Marlhac' and 'Bouche de Betizac', have good resistance to *Phytophthora* fungal infections and are suitable for reproduction with layering and cuttings. Resistance to insect pests have been slightly less investigated, except for the gall wasp (*Dryocosmus kuriphilus*), in which the focus has been on studying the growth of shoots, crown density and the morphology of buds (Bounous, 2005). The cultivar 'Bouche de Betizac' shows tolerance here, if not resistance to this pest. In 2003, a project was started in Piedmont of establishing biological control using the parasitoid wasp (*T. sinensis*) and selection of resistant genotypes. The 'Bouche de Betizac' cultivar was found to be completely resistant to the gall wasp. The resistance mechanism of the 'Bouche de Betizac' cultivar is unknown but it has been found that dormant winter buds can contain eggs or larvae, although the galls are not formed in the spring after the development of buds (Dini et al., 2012). In this case, careful control and integration of trees with natural resistance into plant breeding programs is very promising but progress is very slow.

Anagnostakis et al. (2011) found in the US state of Georgia, that "chinquapins" (chestnuts with a single nut/fruit instead of three, in a thorny wrap) rarely showed symptoms of chestnut gall wasp attack, so they crossed the American chestnut with Ozark chinquapin (*Castanea ozarkensis* Ashe), which was crossed with Chinese chestnut. The authors found that American chestnut and Chinese chestnut are very susceptible to chestnut gall wasp attack and that American chestnut and Ozark chinquapin are susceptible to chestnut blight.

The female parents of the crosses they planted were four different trees of American chestnut, which were half-sisters/half-brothers. The male parents were two distinct trees of *C. ozarkensis* x *C. mollissima* (Ozark chinquapin crossed with Chinese chestnut; the parents of Ozark chinquapin and Chinese chestnut were not the same). Both parents had good resistance to chestnut blight. The descendants of this crossing had some resistance to chestnut blight, which enabled them to survive longer than sensitive genotypes. In addition, it was expected that if resilience to the attack of the gall wasp was inherited easily, some descendants might express some degree of resistance. In 1995, 93 trees were planted in North Carolina, in an area in which the gall wasp was already endemic. In 2009, 36 of these trees had survived, and 31 of them did not have galls (Anagnostakis et al., 2011).

The results obtained in the experiment encouraged the authors, Anagnostakis et al. (2011), to further cross the "chinquapin" chestnut with chestnut species, by selecting trees that were most resistant to the attack of the gall wasp. Resistant trees of Ozark chinquapins, which rarely had galls, began to be used in crosses with the aim of introducing resistance to the gall wasp into commercial chestnut cultivars and interesting hybrid species (Anagnostakis, 2014).

In Japan, selection and breeding began of genotypes that were resistant to chestnut gall wasp within the genetic resources of *Castanea crenata* Siebold & Zucc. and they obtained four resistant varieties. Two of these varieties, 'Tzukuba' and 'Tanzawa', together with the resistant 'Ginyose' variety, are still the most widespread varieties in Japan. Similar studies are under way in the US, where researchers want to introduce new resistance factors to the American chestnut *Castanea dentata* (Marsh.) Borkh. (Dini et al., 2012), using alternative sources of genetic material such as *Castanea mollissima* Blume, *Castanea pumila* (L.) Mill. and *Castanea henry* (Skan)Rehd. & Wils..

Resistance to chestnut gall wasp has also been reported in other species of the genus *Castanea* (*C. mollissima*, *C. pumila*), but not in the variety *C. sativa*. Based on the study of resistance, researchers have indicated that several mechanisms may be responsible for resistance or tolerance in various chestnut genotypes (Dini et al., 2012), which makes breeding work difficult and prolongs the time for obtaining tolerant/resistant varieties.

4 PRESERVATION AND REPRODUCTION OF EUROPEAN SWEET CHESTNUT (*Castanea sativa* Mill.)WITH MICROPROPAGATION

Chestnut is a hard-to-root tree species and grafting is the most common propagation method (Bounous, 2005). Failure to find an effective method of mass reproduction of selected genotypes has suggested micropropagation as a suitable method. The effectiveness of the micropropagation of forest trees depends on the responsiveness of the tissue in *in vitro* conditions, the effectiveness of vegetative propagation of selected varieties, as well as individual trees or genotypes. There are several forest varieties for which the successful establishment of *in vitro* cultures from adult/mature trees has not yet been achieved. Juvenile trees are generally easily reproduced by conventional techniques (Ballester et al., 1990; Ballester et al., 1992; Ballester et al., 1999; Litz, 2005).

Micropropagation, if there is optimization of all phases of work, provides a useful tool for preservation and reproduction of the genetic resources of chestnut. According to the literature data and our experience with Slovenian genotypes, optimization of the rooting phase is very difficult. *In vitro* culture with nodial cuttings was established from young axillary and lateral shoots, mainly from mature though partly also juvenile material. A key factor limiting clonal reproduction of woody varieties is the maturation and age associated non-responsiveness to the formation of roots. Mature and young - juvenile tissues of the same plant source may even have different responses to the added auxin (Pijut et al., 2011). These results can be explained by epigenetic changes during the maturation process. It had been repeatedly reported also for European chestnut, that the success in rooting strongly depends on maturation levels of the stock plant material (Sánchez and Vieitez, 1991; Gonçalves et al., 1998; Corredoira et al., 2017). Moreover, chestnut cuttings (*Castanea sativa*) from mature trees have an elevated level of methylation of DNA compared to cuttings from juvenile trees (Hasbun et al., 2007). After several subcultures obtained by *in vitro* reproduction, some micro-cuttings can root as a consequence of gaining a certain phase of rejuvenation (Pereira-Lorenzo and Ramos Cabrer, 2007).

Studies have shown that the formation of roots is a hereditary quantitative feature, controlled by several endogenous and environmental factors. The most important of these are the juvenility of the stock plant material, hormones, especially auxins, light, temperature and mineral nutrition (Pop et al., 2011; Pacurar et al., 2014).

Physiologically, the process of root development consists of three consecutive but interdependent phases: (1) induction, (2) initiation and (3) expression. The three stages of rooting differ from each other and have different hormonal requirements. Root buds are formed from the cells between the vascular contacts, which accumulate starch for the first 24 hours and the cells become capable of forming root buds between 72 - 96 hours (Pop et al., 2011).

European sweet chestnut (*Castanea sativa*) had been repeated categorised as especially difficult-to-root species via micropropagation. Other chestnut species, including interspecific hybrids (*C. crenata* × *C. sativa*) are much easier to root (Gonçalves et al., 1998; Tetsumura in Yamashita, 2004; Corredoira et al., 2017). Different methods *in vitro* had been tested in the past to improve rooting process in *Castanea* microshoots, including micrografting (Šiftar, 1992). Generally, all methods can be divided into two groups: methods where micro shoots are rooted on medium supplemented with auxin and methods where auxin had been added to micro shoots by dipping their basis into the auxin solution before transferring the shoots into the medium. Gresshoff and Doy (1972) medium is mostly used as a basal medium, some experiments used for rooting of sweet chestnut micro shoots also MS-medium. The majority of the references also reported that the basal medium should contain ½ or ⅓ of the full concentration of macro- and micronutrients when it is used for rooting. Tetsumura in Yamashita (2004) successfully rooted micro shoots of the Japanese chestnut by culturing the shoots on (½) MS-medium supplemented with 15 µM IBA in the dark. Gonçalves et al. (1998) used a combination of Gresshoff and Doy (macronutrients) and MS-medium (micronutrients) supplemented with 3 mg/l IBA for successful rooting of hybrid chestnut (*C. sativa* × *C. crenata*) microshoots. Sanchez and Vieitez (1991) and Vielba et al. (2011) succeeded to root European chestnut by quick-dipping (0.5 – 3 min. or 1 min.) micro shoots in aqueous solution of IBA before transferring the shoots to IBA-free medium. Nevertheless, the micropropagation is still remaining a very doubtful method for economic propagation of European sweet chestnut plants (rooting and acclimatisation problems), but it is a useful method for preservation interesting genotypes (Capuana and Di Lonardo, 2013).

5 CONCLUSIONS

Some interesting Slovenian chestnut genotypes have been recorded in the field but they are recently at risk of decay from chestnut gall wasp, so we decided to preserve them in tissue culture through micropropagation. We have managed to optimize most of the stages of work, except for rooting and acclimatization, which corresponds to the experience of other authors, who have reported that the phase of rooting *in vivo* and *in vitro* of European sweet chestnut (*Castanea sativa* Mill.) is difficult. It also affects plant breeding programs, which are additionally faced with problems in selecting the best parents from a variety of available genetic resources and with incorporating resistant genes into European genotypes. Limiting factors also include high heterozygosity, a long juvenile period and a lack of markers for the early selection of tolerant/resistant offspring. All of this causes a lack of elite planting material for the establishment of new chestnut crops and the replacement of damaged trees in forest stands.

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