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The use of tissue culture methods for producing resistant material of *Aesculus hippocastanum*

– A literature study

Användningen av vävnadsodlingstekniker för produktion av resistent material av Aesculus hippocastanum – En litteraturstudie

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Abstract

The horse chestnut *Aesculus hippocastanum* is a European species of broadleaf trees classified as vulnerable. The species is subjected to many pathogens and pests threatening its survival. Resistance to some pathogens of horse chestnut has been investigated and tissue culture methods such as somatic embryogenesis have been successful. Several tissue culture methods exist to produce plantlets *in vitro* by different micropropagation techniques such as somatic embryogenesis and meristem culture. Somatic embryogenesis has been used for propagation of *A. hippocastanum* in the past. There have also been studies made for producing resistance in *A. hippocastanum*. Several tissue-culture methods have been used for the preservation and promotion of different plant species and they could possibly be eventual approaches for preserving the horse chestnut and produce resistant material. It is concluded in this paper that tissue culture techniques might be of possible use to produce resistant individuals of *A. hippocastanum*, but that it is hard to know for certain, since previous studies for promotion of resistance by tissue culture have been made on different species.

Keywords: *Aesculus hippocastanum, Resistance, Tissue culture, Micropropagation, Somatic embryogenesis*

Sammanfattning

Hästkastanjen *Aesculus hippocastanum* är en europeisk art av lövträd som är klassificerad som sårbar. Arten är utsatt för många patogener och skadedjur som hotar dess överlevnad. Resistens mot några av dessa patogener hos hästkastanj har undersökts och vävnadsodlingsmetoder som somatisk embryogenes har använts med framgång. Flera vävnadsodlingsmetoder finns för produktion av plantor *in vitro* med olika mikroförökningstekniker som till exempel somatisk embryogenes och meristemkultur. Somatisk embryogenes har använts för förökning av *A. hippocastanum* förr. Det har även gjorts studier för framtagande av resistens hos *A. hippocastanum*. Flera olika vävnadsodlingsmetoder har använts för bevarande av och framtagande av andra växtarter, vilket skulle kunna vara potentiella metoder för bevaring av hästkastanjen och produktion av resistent material hos arten. Slutsatsen i denna uppsats är att vävnadsodlingstekniker skulle kunna vara användbara för att producera resistenta individer av *A. hippocastanum*, men det är svårt att veta säkert då tidigare studier för resistensframtagande via vävnadskultur har gjorts med andra arter.

Nyckelord: *Aesculus hippocastanum, Resistance, Tissue culture, Mikroförökning, Somatisk embryogenes*

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Abbreviations

PGR	Plant growth regulators
SLU	Swedish University of Agricultural Sciences
IUCN	International Union for Conserving Nature
IBA	Indole butyric acid
2,4-D	2,4 dichloro-phenoxy acetic acid
BAP	6-furfyrile-amino purine
MS	Murashige & Skoog
PAMP	Pathogen associated molecular patterns
PTI	PAMP triggered immunity
PRR	Pathogen recognition receptors
ABA	Abscisic acid

Purpose of literature study

The purpose of this literature study was to examine if/how tissue culture methods could be used as a tool to combat the decline/threats from pathogens and pests in horse chestnut.

Questions asked:

- "What pathogens and diseases are affecting the horse chestnuts and how?"
- "What methods could be used to reproduce resistant individuals to combat pathogens and pests?"
- "Are tissue culture-based methods possible to be used as a method of multiplying disease resistant cultivars of horse chestnut?"

Limitations

Impacts by several pathogens and pests, especially the most damaging and common ones, are investigated. The most used practice of tissue propagation is described and discussed in relation to previous research on the subject.

1. Introduction

The horse chestnut, *Aesculus hippocastanum*, is a large deciduous ornamental tree in the family Sapindaceae. The genus *Aesculus* contains 12-13 species which are restricted to North America, Europe and south east Asia (Thomas *et al.* 2019).

In Europe the horse chestnut has become subject to many different pathogens that have spread rapidly in European populations, some of which can be lethal. Because of this and other threats the species is now classified as vulnerable by the IUCN and there is a need for measures to prevent the decimation of European horse chestnut populations (Allen, D.J. & Khela, S. 2017).

Resistant or tolerant individuals have been investigated, as in the example of *Pseudomonas syringae pv aesculi*, where studies tested progeny from individuals that previously showed potential resistance (Pánková *et al.* 2015).

There have been studies investigating the use of tissue culture, the use of plant explants grown on synthetic medium in a controlled environment, for reproducing horse chestnuts *in vitro*.

In relation to the horse chestnut's status as vulnerable, there are examples from when tissue culture methods have successfully been used to help multiply and preserve endangered tree species from other parts of the world (Bunn *et al.* 2011), and thus might be a possible tool for multiplying and preserving horse chestnut individuals with resistance properties.

1.1. Aesculus hippocastanum

The horse chestnut has been in cultivation for 400 years, owing its popularity to the shady foliage and candlelike beautiful flowers. It´s native origin is south east Europe, but it has been introduced to most of western Europe. At maturity this tree can reach a height of 36 m and a width of 25 m with a trunk diameter of 2,2 m (Moore & White 2013).

The bark of young individuals starts off as smooth dark grey which changes into a scaly texture as the tree ages. Typical feature of older trees are branches first growing in an upwards fashion, then down and finally up again. The horse chestnut has large 2,5 – 5 cm brown buds usually covered in a sticky resin. Its palmate leaves are up to 60 cm wide with 5 – 7 leaflets per leaf, each 8-20 cm long. These leaflets are joined together at the tip of the petiole which is 7 – 20 cm in length. The inflorescences are 20 cm upright panicles which are either conical or cylindrical androgynous with male flowers in the top and hermaphrodite in the bottom. The individual flowers are zygomorphic with sepals in a tubular shape and 4 – 5 petals, 5 – 9 stamens and a single style (Thomas *et al.* 2019). These inflorescences usually appear after 6 – 8 years of growth. Horse chestnut trees normally reach an ultimate age of 150 years (Moore & White 2013).

Figure 1, A horse chestnut (Aesculus hippocastanum) tree (Photo: Johan Pettersson 2020)

1.2. Pathogens on *A. hippocastanum*

1.2.1. *Pseudomonas syringae*

The bleeding canker disease is caused by the gram-negative bacterium *Pseudomonas syringae pv. aesculi*. It was first discovered on Indian horse chestnut, *Aesculus indica*, according to Durgapal (cited in (Steele *et al.* 2010)) and is thought to have been imported into Europe by contaminated plants (Brasier 2008). It is currently causing a wide outbreak of "bleeding canker disease" on horse chestnuts in many parts of Europe. A visible symptom caused by the infection of *P. syringae* is an oozing rust-brown liquid seeping from cracks in bark (as seen in figure 2), on main stems and branches. The infection in *A. hippocastanum* often leads to dieback and the eventual death of the tree (Green *et al.* 2009). Inside the tree, cankers are created in the form of lesions that develop in the phloem and cortex, which extend into the cambium. This canker formation can establish quite quickly and according to Steele et al(2010). extensive canker formation in the phloem and cambium can develop within a year. It has been suggested that the quick spread of *P. syringae pv. aesculi* might be due to the ability to infect through aerial parts. Observations have shown that *P. syringae pv. aesculi* infects the trees via stomata, lenticels and leaf scars during summer and autumn (Steele *et al.* 2010). Research has shown that *P. syringae* has 2 stages of growth on host plants, namely the epiphytic stage and the endophytic stage. In the epiphytic stage of *P. syringae*, it lives on plant parts above the soil, such as fruits, leaves and stems. In the endophytic stage *P. syringae* enters the apoplast of the host where it multiplies, and without programmed cell death of the infected cell this happens more aggressively. Infected cells die at the end of pathogenesis which results in extensive necrosis (Xin & He 2013).

Figure 2. Bleeding canker caused by Pseudomonas syringae, (F. Lamiot 2006) CC BY-SA

1.2.2. *Phytophtora ssp*

Phytophtora is a genus of fungal-like organisms belonging to phylum Oomyceta within the kingdom *Stramenopila*. The impact from *P. ssp* has worsened as many new pathogens have arisen and many new species of *P. ssp* have been discovered during the last 10 years (Redondo 2018).

The many species of *P. ssp* often have a wide range of host species. There are several ways of infection, either by roots, branches or through leaves. But most species are soil-borne, infecting the fine roots of trees by zoospores, which can be seen in Figure 2. These zoospores are chemotactically attracted to roots where they encyst and penetrate root tissue by hyphae which infect cells and form intracellular haustorium. Infection in susceptible hosts escalates by hyphae invasion of phloem where it causes damage. Xylem more rarely becomes infected but can quickly result in death of infected trees. Severe infection also results in the formation of resting spores (Oßwald *et al.* 2014).

P. ssp are hemibiotrophic and initially lives on host biotrophically after initial infection but later becomes necrotrophic and killing host tissues. The most striking symptom of *P. ssp*

infection are bleeding sap from wounds in the bark (Figure 3), though this is not always the case. Many species also cause root rot which results in dieback in the aerial parts of trees (Oßwald *et al.* 2014).

Several species of *Phytophthora* have been found on horse chestnuts. One recent example is *Phytophthora citrophthora* which was discovered on horse chestnut in Turkey. This was revealed through dieback symptoms and lesions on older trees, and *P. citrophthora* was investigated to have a 40 % mortality on horse chestnut saplings (Akıllı *et al.* 2012). Another example pf *Phytophtora* plaguing horse chestnut is *Phytophtora cactorum,* which causes reddish necrotic lesions on bark that releases oozing liquid and abnormally small leaves of the foliage (Intini *et al.* 2002).

Figure 3, Bleeding canker possibly caused by Phytophthora (RHS/Plant Health 2009) © RHS,

1.2.3. *Cameraria ohridella* - Horse chestnut leafminer

One of the most widespread and easily noticeable pests on horse chestnut is the horse chestnut leafminer; *Cameraria ohridella*. It is a moth in the Gracillariidae family, member of the order Lepidoptera. Its larvae stage (see figure 3) mines the leaves of horse chestnuts and causes browning of the foliage (Pocock & Evans 2014). Mines between the two epidermal layers develop inside the leaf by larvae feeding on the parenchyma cells which causes physical destruction of leaf tissue and results in stunted growth and reduces the amount of photosynthetically active tissue available (Thalmann *et al.* 2003).

Affected leaves initially display brown spots on the leaf surface which quickly spreads through the rest of the foliage. By July/August the leaves of *A. hippocastanum* show an autumn-like appearance and eventually fall of the tree prematurely. It is considered a cosmetic pest since *C. ohridella* seemingly doesn't cause crown dieback and infected individuals sprout new leaves every year. The moth develops 2-4 generations per year and during winter the moth hibernates in fallen leaves on the ground (Thalmann *et al.* 2003).

Studies from Germany have shown that infestation by *C. ohridella* affects the reproduction of *A. hippocastanum* by decreasing dry weight of seeds by almost half in heavily infested trees. This loss of dry weight in seeds may reduce survival of seedlings and threaten the remaining wild populations of the species (Thalmann *et al.* 2003).

Figure 4, Cameraria ohridella larvae (Bower 2006), CC BY-NC-SA 2.0

1.2.4. *Guignardia aesculi* – Horse chestnut leaf blotch

The leaf blotch fungus, *Guignardia aesculi*, is a pathogen in the Botryosphaeriaceae family. This species of sac fungi is not only restricted to *A. hippocastanum*, but occurs on many other species of the genus *Aesculus* as well. It is widespread all cross of Europe (Pastiráková *et al.* 2009).

The fungus matures in fallen leaves in spring and releases ascospores which create the primary infection of tree foliage. Visible infection shortly appears as water-soaked blotches that enlarge and turn reddish brown (Figure 4). The outer edges of these blotches take on a yellow color that separates reddish tissue from green healthy tissue. Black pycnidia of the conidial state of *G. aesculi*, *Phyllosticta sphaeropsoidea*, form shortly after blotches and develop dark subepidermal hyphae which penetrate host cells by forming appressorium which then allows hyphae to penetrate the epidermal cells and grow both intra- and intercellularly, spreading quickly within leaf tissue. In comparison to other pathogens, this does however not happen via stomata. Pycnidia produce macroconidia which are released to create new waves of foliar infection throughout summer. In late summer, *Asteromella aesculicola*, the microconidia anamorph of this fungi, appear and produces spores which mature on fallen leaves to start a new wave of infection the following year (Kopačka & Zemek 2012).

Figure 5. Sympotoms caused by Guignardia aesculi on horse chestnut leaf, (LennyWorthington 2007) CC BY-SA 2.0

1.3. Tree resistance

Resistance is an inherited ability of a plant to escape attacking enemies like pests, pathogens or herbivores in order to minimize damage inflicted (Stenberg). Means of defense in plants can be divided into 2 different categories, biochemical reactions for production of substances and structural barriers (Agrios 2005).

Biochemical defenses are the production of substances or toxins that either kill or inhibit an attacking pathogen, these can either be preexisting or induced. One example of preexisting biochemical defenses is the presence of inhibitory substances such as fungitoxic exudates that can diffuse out onto the surface of a plant and inhibits the germination of spores. Cells already contain phytoanticipins ahead of infection which prevent and inhibit pathogen growth. Other compounds can have membranolytic properties on attacking pathogens which destroys the cell membrane of the pathogen (Agrios 2005).

PAMPs (pathogen associated molecular patterns) are molecules recognized by plants via PRRs (pathogen recognition receptors). PAMP recognition can induce PTI (PAMP triggered immunity) in plants through recognition by PRRs during parthenogenesis. A study has shown that chitosan, a naturally occurring compound, can act as PAMPs by increasing the activity of defense proteins as well as ABA (Abscisic acid) levels in plant cells which reduces the impact of diseases(Kuyyogsuy *et al.* 2018).

Structural defenses or barriers can also be divided into preexisting and induced, where preexisting can be the outer wax coating of the epidermis which prevents the formation of water film for growth of fungi or bacteria. This may also prevent pathogens from penetrating the epidermal cell layer. Thick outer cell walls are another preexisting structural defense which helps prevent infection since pathogens have a harder time penetrating such cell walls.

In induced structural defenses the pathogen is detected by the host plant through signal molecules released from a pathogen. These are often compounds released when a pathogen breaks down host cells or vice versa. Once the pathogen is detected it initiates structural changes of the host organisms' cells to combat the pathogen. One of these is the swelling of the outer cell wall which creates a fibrous material trapping pathogen on the surface. Another is the thickening of - or deposition of phenolic compounds into attacked cell walls. There are also

histological responses such as the formation of cork tissue which prevents the spread of pathogens and restricts nutrient flow in infected areas. If the infected tissue is located on leaf structures the plant can effectively remove this by forming an abscission layer of the infected leaf, and thus disposing of the pathogen (Agrios 2005).

1.4. Tissue culture

The practice of tissue culture is the culturing of cells or organs from plants under controlled environments and aseptic conditions. The medium used contains the necessary nutritional elements such as vitamins, sugars, amino acids, micro- and macroelements for the growth or regeneration of plant tissues. Plant growth regulators (PGR) are often added to induce the development and growth of the plant tissues *in vitro* (Satbir & Wani 2018).

The most important PGRs are auxins and cytokinins. Commonly used auxins are indole butyric acid (IBA) for cell division, the induction of cell elongation and rooting, or 2,4 dichloro-phenoxy acetic acid (2,4-D) which induces formation of undifferentiated callus cells. The cytokinin 6-furfyrile-amino purine (BAP) induces the formation of buds/shoots and an increase in cell division (Satbir & Wani 2018).

When culturing explants by tissue culture they de-differentiate, meaning that they revert from mature stage and become meristematic. The resulting callus mass of undifferentiated cells can be used in inducing root or shoot formation and somatic embryos through organogenesis (Satbir & Wani 2018).

Tissue culture has some advantages compared to the use of traditional plant propagation methods such as by cuttings or seeds. Seeds and shoots are not always readily available all year around while tissue culturing of shoots can be performed at any given time. Seeds and cuttings also deteriorate quickly in many cases. Depending on species, seeds may lose viability after some time. In the case of cuttings, tissue culture has the advantage of not harming the plant, which is subject to propagation, while cuttings require the physical removal of plant parts. In the case of species where the total number of individuals is limited, removing the plant or parts of it might endanger the survival of the species. Taking small samples for tissue culture is less risky. Materials taken for tissue culture also has the benefit of being easily exchanged internationally, with less restrictions (Cruz-Cruz *et al.* 2013).

1.4.1. Micropropagation

Micropropagation is a tissue culture technique defined as the controlled *in vitro* culturing of small explants under controlled conditions in order to produce many plantlets (Satbir & Wani 2018). The main purpose of micropropagation is to produce identical or clonal microplants which are true to type (Edwin *et al.* 2008).

There are several techniques to produce microplants but in general there are three different ways. The first one is the process of somatic embryogenesis in which embryos are formed *in vitro* and allowed to germinate into plantlets. The second one is by inducing the formation of adventitious shoots from any kinds of explants through *de novo* meristem formation via callus or directly from the explants. The third way is by letting shoot buds or meristems grow into plantlets (Edwin *et al.* 2008).

The process of micropropagating plants proceeds by 5 steps. First, explants are taken from *in vivo* grown individuals, often from shoot apices, meristems or nodal segments. These explants are surface sterilized and grown on medium until shoots/roots begin to form (Satbir & Wani 2018). Secondly, the shoots and buds are multiplied. Previous cultures are divided into smaller parts and transferred onto medium containing PGR, like cytokinin and auxin, which results in rapid cell division. In this stage cytokinin concentrations are generally high, thus shoot (not root) formation is stimulated. Thirdly is the induction of roots and hardening. Plantlets are cultured on medium containing auxins like IBA to induce the formation of root primordia. This is followed by cleaning of plantlets and transfer to a new rooting medium in greenhouse. The greenhouse environment provides the necessary environment for acclimatizing of plantlets. Fourthly, the acclimatized plantlets are transferred for rooting in soil which is also done in greenhouse environment. The fifth and last step is to transfer successfully established plantlets into the field for further growth (Satbir & Wani 2018).

1.4.1.1. Somatic embryogenesis

The process of embryogenesis in plants normally occurs during the process of double fertilization. In double fertilization the haploid sperm cell fuses with the haploid egg cell to give rise to a diploid embryo which becomes a new individual. The process occurs *in vivo* in the flowering organs in the case of angiosperms and results in seed formation. But the same process of embryogenesis can also occur asexually through the asexual formation of seeds (Germana & Lambardi 2016).

The process of somatic embryogenesis is the formation of embryos from one or a group of somatic cells *in vitro* using plant growth regulators (PGR) in order to induce the embryogenic state (Germana & Lambardi 2016). It is based on totipotency – any cell, theoretically, can give rise to a whole new individual (Rocha 2013). Somatic embryogenesis is divided into 2 phases, the induction phase and expression phase. Induction phase is initiated by exogenous application of PGRs through which the somatic cells attain the embryonic stage and become embryonic cells. After this the expression phase is induced where the now embryonic cells develop into an embryo. Auxin is a PGR which is known to induce embryogenesis and the most commonly used for this purpose. However, abscisic acid and cytokinin are also known to induce embryo formation (Germana & Lambardi 2016).

Somatic tissues are taken as explants from tissues like leaves which are wounded and sterilized. After exogenous adding of PGR, the embryogenic cells/explants are cultured on a medium free from PGR (Germana & Lambardi 2016).

1.4.1.2. Meristem culture

Meristem culture is another type of micropropagation that utilizes the apical meristems as explants to produce virus free plantlets. Apical meristems remain free from viruses, bacteria and fungi because connective tissues do not form until later in a plant's development (Mori & Hosokawa 1977). Disinfected explants are cultured on media and then tested for virus content by electron microscopy. These cultured explants are further cultured by micropropagation to produce new disease-free plants (Satbir & Wani 2018).

1.5. Inoculation tests for susceptibility in *A. hippocastanum*

One study where inoculation with *P. syringae* was tested on progeny from parental trees of *A. hippocastanum* with potential resistance showed that 40 % of 322 tested progeny were resistant, while 40 % became tolerant after 2-3 years of growth. Only 20 % of progeny appeared to be susceptible to *P. syringae* and 70 % of the tolerant progeny revealed the survival of *P. syringae* in tissues of progenies, however these showed no bleeding canker symptoms. This study further concluded that there is naturally occurring resistance in horse chestnut populations that could be used for producing resistance (Pánková *et al.* 2015).

1.6. Tissue culture methods applied on horse chestnut

Previously, application of tissue culture methods has been applied on *A. hippocastanum*. Studies in the past have shown successful plant regeneration of horse chestnut through somatic embryogenesis by using immature embryos to create callus culture. This was performed using Murashige and Skoogs (MS) medium (Murashige & Skoog 1962) (Radojevic 1988). Further additional studies have investigated the usage of secondary somatic embryogenesis, which is the initiation of somatic embryos from other primary somatic embryos which has potential for mass clonal propagation (Calic *et al.* 2005).

1.6.1. Examples from other species

Eucalyptus marginata

There are other cases where tissue culture has been used to produce resistant material to pathogens and pests. One such case is the micropropagation of *Eucalyptus marginata*, an important timber production tree, for producing resistance to *Phytophtora cinnamoni*. This study demonstrated how plants of *E. marginata* were inoculated with *P. cinnamoni* and thereafter evaluated based on developed lesions. The ones with less lesions were then selected and tissue sampled for tissue culturing, multiplied *in vitro*, established and transferred for field trails. Survival of resistant clones were higher than that of susceptible. The study further concluded that selection of resistant clones is a good method for producing resistant individuals (Stukely *et al.* 2007).

Castanea sativa

Another study investigates the micropropagation of *Castanea sativa* for resistance to *P. cinnamoni.* Tests on micropropagated plantlets via inoculation with the pathogen were performed both through stem and substrate to test for resistance. Substrate inoculation more closely resembles the natural infection pathway of *P. cinnamoni*. This study produced several clones of *C. sativa* that were resistant to *P. cinnamoni* (Cuenca *et al.* 2010).

Juglans ssp

Resistance to *Phytophthora cinnamoni* and *Phytophthora citricola* has been investigated using micropropagation on various species of walnuts, including some hybrids, where explants from nodal stem cuttings were taken and cultured on media to produce plantlets. Cultured plantlets transferred to substrate were tested by inoculation to examine the resistance to *P. cinnamoni* as well as *P. citricola* and clones of one walnut hybrid were revealed to exhibit resistance towards phytophthora (Browne *et al.* 2015).

2. Method

2.1. Literature study

This thesis has been made in the form of a literature study based on already existing research reports. Information gathering was conducted systematically using databases/search engines such as “Google Scholar” and “Primo, SLU database”. The local library on campus in SLU Alnarp was used as well in order to find literature sources published in books or journals.

Examples of phrases used in the search for sources were “*Aesculus hippocastanum*”, “*Pseudomonas syringae*”, “*Cameraria ohridhella*”, “Tissue culture methods”, “Somatic embryogenesis” and “auxin”. Sources have been selected based on relevancy for the subject and time of publishing. More recent sources were prioritized but since some information on the subject was scarce, older sources were also used.

3. Discussion

The severity of the different pathogens plaguing *A. hippocastanum* seem to vary in impact and severity on tree growth and development. If for instance, *C. ohridella* causes a reduction in photosynthesis and reduced reproduction, which can seriously affect trees, while. *P. syringae* might be seen as a more acute threat since it could have lethal outcomes for horse chestnut trees. The same goes for *Phytophtora* which also seems to be a more severe pathogen compared to *G. aesculi.*

As mentioned earlier the use of meristem culture has the advantage of eliminating viruses (Mori & Hosokawa 1977), bacteria and fungi by culturing cells from apical meristems. The scope of this paper is to investigate the promotion of resistance through tissue culture and has been the focus. However, the aspect of producing plantlets that are free from pathogen from the very beginning might be interesting. Meristem culture might work as a tool for achieving this by eliminating bacteria such as *P. syringae* and fungi such as *G. aesculi. Phytophtora* however is a oomycete (Oßwald *et al.* 2014) and therefore it cannot be said if this would be possible, since it is hard to say if oomycetes are spread to meristem cells or not.

Examples given for other species when it comes to tissue culture for producing resistant material may or may not be applicable methods for horse chestnut, though that is not certain since the examples concern completely different species of different genus.

While it is hard to say which method of tissue culture could be used based on the information gathered in this paper but according to the previously made attempts used on *A. hippocastanum* (Radojevic 1988; Calic *et al.* 2005), it seems like somatic embryogenesis is a viable method for tissue culturing horse chestnuts, although none of these examples seem to include resistance. Furthermore, the study made to investigate resistance to *P. syringae* (Pánková *et al.* 2015) utilizes similar methods of inoculation tests as in previous examples for other species. Maybe this, in combination with the somatic embryogenesis with horse chestnut, could be a possible approach to produce resistant material similarly to how it was made for *E. marginata, C. sativa and Juglans ssp* (Stukely *et al.* 2007; Cuenca *et al.* 2010; Browne *et al.* 2015). But these were studies made with different techniques and one important point brought up in several other examples of tissue cultured plants (Bunn *et al.* 2011) is that not all techniques work on every species of plant, which might be important to take into consideration.

3.1. Conclusion

There are many pathogens and pests which are attacking the horse chestnut, even if not all are equally damaging or lethal. Somatic embryogenesis has been used for propagation of *A. hippocastanum* in the past. There are several tissue culture approaches which have been used for the preservation of different plant species and they could possibly be eventual approaches for preserving the horse chestnut and producing resistant material. Several examples exist on how tissue culture methods have been used to produce resistant material in other plant species, however it is hard to say for certain if these approaches would be possible for horse chestnut. It is however worth examining this for the horse chestnut.

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