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Comparative study of different surface sterilization treatments and optimal month for establishment of aseptic cultures of raspberry cultivars

Uporedna analiza različitih tretmana za sterilizaciju i optimalnog meseca za uspostavljanje aseptičnih kultura sorti maline

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ABSTRACT

Removing contaminants from plant material with minimal damage to plant cells using different chemical solutions represents very important and one of the most critical steps in establishing plant tissue culture *in vitro*. Our study deals with the optimisation of the protocol for successful surface sterilization of initial explants in two raspberry cultivars, 'Meeker' and 'Willamette' in two experimental years. The main objective during the first experimental year was to examine the protocols for surface sterilization which included different combinations of disinfectants (mercuric chloride or commercial bleach solution each in combination with 70% ethanol). In the following year, the impact of the two most effective sterilization treatments on the establishment of aseptic cultures over the four-month period (May, June, July, and August) was investigated in both raspberry cultivars. Aseptic cultures were established on Murashige and Skoog medium (MS) containing 2.0 mg/l N6-Benzyladenine (BA), 0.5 mg/l indole-3-butyric acid (IBA) and 0.1 mg/l gibberellic acid (GA_3). The highest percentage of explants initiation in 'Meeker' (79.3%) was achieved with surface sterilization in 70% ethanol (1 min) combined with 10% (v/v) commercial bleach solution (20 min), whereas sterilization in 70% ethanol (1 min) followed by $HgCl_2$ (3 min) gave the best results (38.3%) in 'Willamette'. The most optimal month for the establishment of aseptic culture was May for 'Meeker', while June was the most suitable month for the 'Willamette' cultivar.

Keywords: *Rubus idaeus*, tissue culture, disinfectants, aseptic culture establishment

SAŽETAK

Eliminacija zaraze sa biljnog materijala uz minimalno oštećenje biljnih ćelija primenom različitih hemijskih rastvora je izuzetno važan i jedan od najkritičnijih koraka u postupku uspostavljanja *in vitro* kulture biljnog tkiva. U radu se ispituje optimizacija protokola uspešne površinske sterilizacije početnih eksplanata kod dve sorte maline, 'Meeker' i 'Willamette', tokom dve eksperimentalne godine. U prvoj eksperimentalnoj godini, ispitivan je protokol za uspešnu površinsku sterilizaciju, uključujući varijacije dezinficijenata poput živinog hlora i varikine, svaki u kombinaciji sa 70% etanolom. Sledeće eksperimentalne godine, ispitivan je uticaj dva najefikasnija tretmana sterilizacije na uspešnost uspostavljanja aseptične kulture tokom četvoromesečnog perioda od maja do avgusta meseca. Aseptična kultura je uspostavljena na Murashige i Skoog podlozi (MS) koja sadrži 2.0 mg/l N6-benziladenina (BA), 0,5 mg/l indol-3-buterne kiseline (IBA) i 0,1 mg/l giberelinske kiseline (GA_3). Najviši procenat inicijacije eksplantata kod sorte 'Meeker' (79,3%) postignut je pri površinskoj sterilizaciji u 70% etanolu (1 min) u kombinacijii sa 10% (v/v) varikinom (20 min), dok je sterilizacija u 70% etanolu (1 min) praćena $HgCl_2$ (3 min) dala najbolje rezultate (38,3%) kod sorte 'Willamette'. Najoptimalniji mesec za uspostavljanje aseptične kulture bio je maj za sortu 'Meeker', dok je jun najpogodniji mesec za sortu 'Willamette'.

Ključne reči: *Rubus idaeus*, kultura tkiva, dezinfekciono sredstvo, uspostavljanje aseptične kulture

INTRODUCTION

Raspberry (*Rubus ideaus* sp.) holds an important position in the overall fruit production in the Republic of Serbia as it is economically one of the most important berry fruits (Mitrović et al., 2023). The most common raspberry cultivar grown is 'Willamette' accounting for more than 90% of plantations, followed by 'Meeker' with 3–5% (Karaklajić-Stajić et al., 2023). In recent years, the major problem in raspberry production and cultivation is drying caused by pathogens that are transmitted by planting material. Therefore, it is necessary to use healthy, physiologically uniform and true-to-type planting material of high quality for establishment of new orchards (Petrović et al., 2017; Melyan et al., 2021). The Fruit Research Institute in Čačak has begun controlled production of raspberry planting material by tissue culture *in vitro*, using nuclear stock produced according to the Certification scheme for *Rubus*. Micropropagation of raspberries represents a unique way to produce large quantities of high-quality and disease-free plants in a very short period. *In vitro* propagation includes four different stages: establishment of aseptic culture, multiplication, rooting and acclimatization of the *in vitro* plants. Plant tissue cultures are initiated from tiny pieces, called explants, taken from any part of a plant. Although any part of a plant can be used successfully as a source of explants, shoot-tips, meristem-tips and nodal buds (axillary buds) are mainly used as initial explants to initiate aseptic culture.

Surface sterilization of plant materials followed by leaf rosette initiation is critical step in establishing plant tissue culture protocol. Successful tissue culture of any plant species depends on the removal of exogenous and endogenous contaminating microorganisms (Sehrawat et al., 2021). According to Ayele and Tefera (2018) a good clean explant, once established in an aseptic condition, can be multiplied several times. However, the initiation of explants under aseptic conditions should be considered a critical step in micropropagation. Namely, explants often fail to establish and grow, not because of the lack of a suitable medium but because of contamination. Surface sterilization of explants is a process that includes the immersion of explants into appropriate concentrations of disinfectant(s) for a specified time resulting in the establishment of a contamination-free culture (Bello et al., 2018). The main problem in achieving this goal is the occurrence of contamination caused by microorganisms.

According to Gogoi and Borua (2014) the main surface sterilizing disinfectants conventionally used in tissue culture laboratories are ethanol (EtOH), sodium hypochlorite (NaClO), calcium hypochlorite [$Ca(OCl)_2$] and mercuric chloride ($HgCl_2$). They are used to reduce contamination, while wetting agents, such as Tween 20 or 80, can be added to disinfectants to reduce surface tension and allow better surface contact (Mahmoud and Al-Ani, 2016). EtOH is a potent and most commonly used sterilizing disinfectant but it is also extremely phytotoxic (Singh et al., 2022). Therefore, the plant material can be exposed to EtOH for a few seconds to a minute. Generally, 70% EtOH is used before treatment with other compounds. Chlorine and chlorine-based sterilizing disinfectants (Na-hypochlorite, Ca-hypochlorite, etc.) generate so-called free chlorine, which destroys microorganisms but also creates very dangerous and undesirable by-products (mutagenic compounds, etc.). The sterilizing effect of hypochlorite dissolved in water is based on production of hypochlorous acid (HOCl), which is a strong oxidizing agent but also a weak acidifier (Kurtović et al., 2016). $HgCl_2$ is also extremely toxic to plants and humans, so proper concentration and duration of exposure are important when used as a surface disinfectant. The optimal concentration which should be used for sterilization is 0.1% for 3, 4 or 5 minutes. Afterwards, the explants must be carefully washed with sterile distilled water to remove the traces of $HgCl_2$. Surface sterilization with $HgCl_2$ (0.1%) for 4 min was the optimal duration, which resulted in the highest percentage of explant survival in strawberry 'Senga Sengana' (Jan et al., 2013). Nacheva and Ivanova. (2017) discovered that silver nitrate ($AgNO_3$) can be used as a disinfectant to control contamination in woody plants.

However, it is very toxic and should be used with great caution. Mihaljević et al. (2013) demonstrated that $AgNO_3$ at a concentration of 1% for 20 minutes was the best sterilizing disinfectant to control contamination in 'Oblačinska' sour cherry.

The first experiments for *in vitro* cultivation of raspberries were carried out in the 1970s and early 1980s (Anderson, 1980). Micropropagation of raspberry using nodal segments or an axillary branching system has been achieved with good results so far (Hunkova et al., 2016). Nevertheless, it remains difficult to establish standard sterilization procedures that can be applied to different genotypes. Examining four raspberry cultivars Georgieva et al. (2020) determined that the genotype plays a crucial role in the success of micropropagation. The concentration and composition of sterilizing disinfectants should be adjusted to achieve maximum sterilising effect with minimal damage to explants. Therefore, proper sterilization of explants is the most essential step for successful propagation in plant tissue culture. To determine the most effective protocol for sterilizing initial explants, the efficacy of two disinfectants ($HgCl_2$ and a commercial bleach solution), each in combination with 70% EtOH, was tested to assess their contamination control activity in two floricane-fruiting raspberry cultivars, 'Willamette' and 'Meeker'. In this research, we experimented with different treatment durations and the timing (month) of harvesting axillary buds to be used in tissue culture establishment.

MATERIALS AND METHODS

Plant material

Experiments were performed with two cultivars, 'Meeker' ('Willamette' × 'Cuthbert') and 'Willamette' ('Newburgh' × 'Lloyd George'). Plants that were used for the experiment are of the pre-basic category produced at the Fruit Research Institute (Jevremović and Paunović, 2010). All plants were maintained in the screen house and tested for the presence of viruses listed in the EPPO certification scheme for *Rubus* (OEPP/EPPO, 2009).

Establishment of aseptic culture

First experimental setup

During the first experimental year, new cultures of both genotypes were established using lateral buds harvested from actively growing primocanes. To initiate an aseptic culture, during a four-month period (May-August), canes were cut into single-node cuttings, leaving 0.5 cm on each side of the axillary bud. The explants were washed under running tap water (1.5–2 hours) and then surface sterilized with $HgCl_2$ or 10% (v/v) commercial bleach solution (0.5%, w/v, final concentration of NaClO) each in combination with 70% EtOH. We examined the effect of seven different treatments (Table 1): 70% EtOH (30 sec or 1 min) in combination with 10% bleach solution (15 and 20 minutes) or with $HgCl_2$ (3, 4 and 5 minutes). The following parameters were monitored: percentage of contamination, necrosis and explant initiation. Initiating explants involves establishing an aseptic culture and initiating the rosette of leaves.

Table 1. Different combinations of disinfectants used for surface sterilization treatment

Treatment	Disinfectant combination
I	70% EtOH (1 min) → 10% bleach (15 min)
II	70% EtOH (1 min) → 10% bleach (20 min)
III	70% EtOH (30 sec) → 0.1% $HgCl_2$ (3 min)
IV	70% EtOH (30 sec) → 0.1% $HgCl_2$ (4 min)
V	70% EtOH (30 sec) → 0.1% $HgCl_2$ (5 min)
VI	70% EtOH (1 min) → 0.1% $HgCl_2$ (3 min)
VII	70% EtOH (1 min) → 0.1% $HgCl_2$ (4 min)

The second experimental setup

In the second year of the trial, once the most efficient sterilization method for explants initiation in both raspberry cultivars was determined, the research then shifted its focus to identifying the optimal month for the establishment of aseptic culture. This phase of the study spanned four months (May, June, July, and August) and

focused on evaluating the impact of the two sterilization procedures (treatment II and VI) on the efficiency of leaf rosette initiation in each month.

After sterilization, explants were carefully washed three times in sterile distilled water, lateral buds were isolated under a stereomicroscope and placed on a basic Murashige and Skoog (MS) nutrient medium (Murashige and Skoog, 1962) containing 2.0 mg/l N6-benzyl adenine (BA), 0.5 mg/l indole-3-butyric acid (IBA) and 0.1 mg/l gibberellic acid (GA_3). The medium contained agar and sucrose at 7 to 20 g/l, respectively. All manipulations were carried out in a laminar airflow hood.

Data collection and statistical analysis

In the first experimental setup, three independent replicates of approximately 20 explants were tested for each of the seven experimental treatments in both raspberry cultivars. Therefore, 420 explants were isolated for each cultivar (7 treatments × 3 replicates × 20 explants). As regards the second experimental setup, each of the two most efficient treatments consisted of 30 explants and all experiments were repeated three times for each of the four months examined (a total of 720 explants per genotype). The following parameters were carefully observed in all experiments: the percentage of contamination, the percentage of necrosis, and the percentage of explants that initiated leaf rosettes. Data presented as percentages were subjected to an arcsine transformation and analysed by ANOVA followed by Duncan's Multiple Range Test for mean separation ($P < 0.05$). Non-transformed data are presented in Tables 2 and 3, and Figures 2 and 3.

RESULTS AND DISCUSSION

One of the first critical steps in *in vitro* propagation is the successful establishment of an aseptic culture. The efficiency of this step is determined by a large number of parameters, of which the timing (the time of the year) and the method of the collection of initial explants, the type of explants, the health and physiological status of the donor plants, the manipulation skills, the procedure of surface sterilization, plant growth regulator composition of medium for rosette initiation and the growth conditions of the initial explants play a key role (Wojtania and Matysiak, 2018; Stanisavljević et al., 2017).

Successful control of contamination depends largely on the disinfectant used and the duration of its exposure. During sterilization, living material should not lose its biological activity and only microbial contamination should be eliminated. Therefore, the plant material must be surface-sterilized with a disinfectant at a suitable concentration and for a specific period of time. In this study, an attempt was made to establish an efficient surface sterilization protocol for *in vitro* propagation of raspberries, using the two most commonly used sterilizing disinfectants ($HgCl_2$ and NaClO) and varying exposure durations of explants. According to Georgieva et al. (2020) a high concentration of ethanol (96%), along with a low concentration of sodium hypochlorite (5%) and short exposure times (30 seconds and 2.5 minutes, respectively), may impact a lower success rate in establishing an aseptic culture, while also leading to a higher contamination rate.

The study also demonstrated that the timing (month) of collecting initial explants significantly influences the success of aseptic culture establishment.

The effect of different surface sterilization procedures on the establishment of aseptic cultures of raspberry cultivars

The different doses of NaClO and $HgCl_2$ showed a significant difference in contamination control, necrosis and aseptic culture initiation in both raspberry cultivars (Table 2).

Among the seven different treatments, treatment II was found to be the most effective method for disinfecting explants from the 'Meeker' raspberry cultivar grown in a screen house. This combination of disinfectants resulted in the highest percentage of aseptic cultures with leaf rosette initiation (79.3%) and the lowest percentages of explant necrosis (3.3%) and contaminated cultures (16.4%; Table 2; Figure 1a).

Table 2. The effect of different sterilization procedures on the establishment of aseptic cultures of the raspberry cultivar 'Meeker'

Treatment	Contaminated cultures (%)	Explant necrosis (%)	Aseptic culture (%)
I	53.4^{b*}	13.3^{bc}	33.3^{b}
II	16.4^{c}	3.3^{c}	79.3^{a}
III	58.3^{b}	23.3^{ab}	18.3^{bc}
IV	50.0^{b}	21.7^{abc}	28.3^{bc}
V	75.0^{a}	15.0^{bc}	10.0^{c}
VI	81.7^{a}	5.0^{bc}	13.3^{c}
VII	26.7^{c}	38.3^{a}	35.0^{b}

* Average values for examined parameters in the same column marked with different letters are significantly different (Duncan Multiple Range Test, $P < 0.05$)

There are many other reports of the successful application of sodium hypochlorite for surface sterilization of initial explants (Abbasi et al., 2017; Al Ghasheem et al., 2018). However, in our study, a shortened (15-minute) treatment with bleach resulted in a significant increase in explants contamination and a decrease in aseptic culture initiation. Compared to bleach as a source of NaClO, $HgCl_2$ was less effective in most treatments, both in terms of the percentage of contamination (which ranged from 26.7% to 81.7%) and in terms of the percentage of leaf rosette initiation (10–35%). The highest percentage of contaminated cultures was obtained with the combinations of 30 sec of 70% EtOH and 5 minutes of $HgCl_2$ (75%), and 1 minute of 70% EtOH and 3 min of $HgCl_2$ (81.7%). Consequently, these treatments yielded the lowest percentages of aseptic culture initiation at 10% and 13.3%, respectively. Surface sterilization with 70% EtOH for 1 minute in combination with $HgCl_2$ for 4 minutes resulted in a similar initiation percentage (35%) as sodium hypochlorite for 15 minutes (33.3%).

Based on the rates of contamination of the aseptic culture initiation in this genotype it can be concluded that $HgCl_2$ should be applied for at least 4 minutes in combination with 1-minute treatment with EtOH to be effective. Relatively high necrosis along with high contamination of axillary buds was noted in *Colocasia esculenta* (Akplogan et al., 2018) and could be explained by the nudity of the axillary buds so that a disinfectant can easily penetrate and provoke cell death and necrosis. The apical buds are protected by primordial leaves, which reduce the penetration of the disinfectant (Akplogan et al., 2018).

In contrast to previous results, treatment II (1 minute of EtOH followed by 20 minutes of NaClO) gave the lowest percentage of aseptic culture initiation (10%) in raspberry 'Willamette'. Generally, $HgCl_2$ proved to be more efficient for surface sterilization of explants in this cultivar (Table 3). The combination of a one-minute soaking in 70% EtOH followed by a three-minute treatment with 0.1% $HgCl_2$ (treatment VI) resulted in the highest percentage of leaf rosette initiated in 'Willamette', at 38.3%. Georgieva et al. (2016) reported a high efficiency of $HgCl_2$ in the surface sterilization of wild small-berry fruits. The combination of 70% EtOH for 1 minute and 0.1% $HgCl_2$ for 5 minutes gave the best results in wild raspberries. $HgCl_2$ at a concentration of 0.1% applied for 3 minutes alone or in combination with 70% EtOH was also found effective for surface sterilization of strawberry cultivar 'Selva' (Kaur et al., 2012) and pomegranate (Guranna and Hoolageri, 2017).

Table 3. The effect of different sterilization procedures on the establishment of aseptic cultures of the raspberry cultivar 'Willamette'

Treatment	Contaminated cultures (%)	Explant necrosis (%)	Aseptic culture (%)
I	58.3^{a*}	16.7^{d}	25.0^{ab}
II	23.3^{b}	66.7^{a}	10.0^{c}
III	55.0^{a}	18.3^{d}	26.7^{ab}
IV	23.3^{b}	46.7^{b}	30.0^{ab}
V	61.7^{a}	20.0^{d}	18.3^{bc}
VI	26.7^{b}	35.0^{c}	38.3^{a}
VII	56.7^{a}	26.7^{cd}	16.6^{bc}

* Average values for examined parameters in the same column marked with different letters are significantly different (Duncan Multiple Range Test, $P < 0.05$)

In our experiments, combinations that involved a quick dip in EtOH for 30 sec followed by a 3- and 4-min application of $HgCl_2$ also yielded satisfactory results, with initiation percentages of 26.7% and 30%, respectively. As for contamination, the results indicated that the percentage of contamination was more affected by the duration of exposure to disinfectants rather than the type of sterilizing disinfectant used. A 20-minute application of sodium hypochlorite resulted in the highest percentage of necrosis (66.7%) which was significantly higher compared to all other sterilization treatments. Considering all parameters of aseptic culture establishment, it is concluded that the most effective sterilization protocol for raspberry 'Willamette' involves a combination of 70% EtOH for 1 minute followed by 0.1% $HgCl_2$ for 3 minutes (38.3%). This combination resulted in the highest percentage of leaf rosette initiation (Figure 1b).

In many studies, it has been proven that a single application of disinfectants provides better results than the combination of different disinfectants. Surface sterilization with $HgCl_2$ (0.1%) for 2 minutes was the optimal duration which resulted in the highest percentage of explants survival in strawberries (Mir et al., 2019). A high percentage of infection in 'Meeker' and 'Willamette' (72% and 74.2%, respectively) was achieved with surface sterilization which included 96% EtOH for 30 seconds followed by 5% NaClO for 2.5 min (Georgieva et al., 2020). The mentioned combination of disinfectants led to a high contamination rate, primarily due to the high concentration of EtOH. Pure alcohol has limited penetration through the cell wall and evaporates rapidly (Dvorak, 2008). In tissue culture, it is commonly used in combination with water because this combination reduces evaporation, increases the contact surface, enhances efficiency, and reduces sterilization time (Quynh et al, 2022).

a)

b)

Figure 1. Fully developed raspberry leaf rosettes after successful establishment of aseptic cultures: a) 'Meeker'; b) 'Willamette'

Evaluation of optimal month for establishing aseptic cultures

In addition to the factors that primarily influence the success of establishing aseptic cultures, such as genotype, type and size of explants, duration of disinfection, and concentration of disinfectant, the season when explants are collected could be a crucial factor. Younger, more rapidly growing tissue or tissue in the early developmental stage has been found to be the most effective (Paunescu, 2009). The study aimed to determine the optimal months for establishing an aseptic culture, considering the influence of two factors: the period of aseptic culture establishment and the sterilization treatment. The tested sterilization treatments included 1 minute of EtOH followed by 20 minutes of sodium hypochlorite, and 1 minute of EtOH followed by 3 minutes of $HgCl_2$. The sterilization treatments (treatment II and VI) were chosen based on their proven effectiveness in the previous experimental year for both cultivars.

The study found that, for establishing an aseptic culture in the raspberry cultivar 'Meeker', the most suitable month was May. During this month, there was a high percentage of aseptic culture initiation (53.3%) with the lowest percentage of explant necrosis (27.2%; Table 4). The highest percentage of contaminated cultures was observed in June (36.7%), while the highest percentage of explant necrosis was recorded in August (98.3%).

When considering the impact of the sterilization treatments as a factor, it was concluded that in raspberry cultivar 'Meeker', the combination that included a dip in EtOH for 1 minute followed by NaClO for 20 minutes (treatment II) produced satisfactory results with 34.2% sterile culture initiation, while the percentage of aseptic cultures with leaf rosette initiation was lower (20.8%) with $HgCl_2$ treatment.

The interaction between the two mentioned factors significantly influenced several key parameters in the 'Meeker' cultivar. Specifically, it had a notable impact on the percentage of contamination, necrotic explants, and explants that successfully formed a leaf rosette. The results obtained from the study demonstrate that the highest percentage of aseptic cultures with leaf rosette initiation was occurred in May, with a value of 60% (Figure 2). This result was observed when using sodium hypochlorite as the treatment for surface sterilization, displaying a notable improvement compared to the treatment involving mercuric chloride, which achieved an initiation percentage of 46.7%. These results align with the results obtained by Tiwari et al., (2002) who also concluded that May is the most optimal month for the success of aseptic culture establishment. In May, both sterilization treatments exhibited significantly lower percentages of necrotic explants compared to other test periods. Notably, the highest percentage of necrosis was recorded in August, reaching 96.7% and 100.0% for treatment with sodium hypochlorite and 0.1% mercuric chloride, respectively. Minimum contamination (19.86%) was observed in May for the establishment of aseptic culture in pomegranate (Kumar et al., 2019) and 8.9% in bamboo (Singhiet al., 2012).

Table 4. Evaluation of optimal month for establishing aseptic culture of raspberry cultivar 'Meeker'

Factors		Contaminated cultures (%)	Explant necrosis (%)	Aseptic culture (%)
Month (A)	May	19.5^{c*}	27.2^{d}	53.3^{a}
	June	36.7^{a}	34.4^{c}	28.9^{b}
	July	28.3^{b}	45.6^{b}	26.1^{c}
	August	0.0^{d}	98.3^{a}	1.7^{d}
Treatment (B)	II	21.7^{a}	44.1^{b}	34.2^{a}
	VI	19.8^{b}	59.4^{a}	20.8^{b}
ANOVA				
A		*	*	*
B		ns	*	*
A x B		*	*	*

* Average values for examined parameters in the same column marked with different letters are significantly different (Duncan Multiple Range Test, $P < 0.05$)

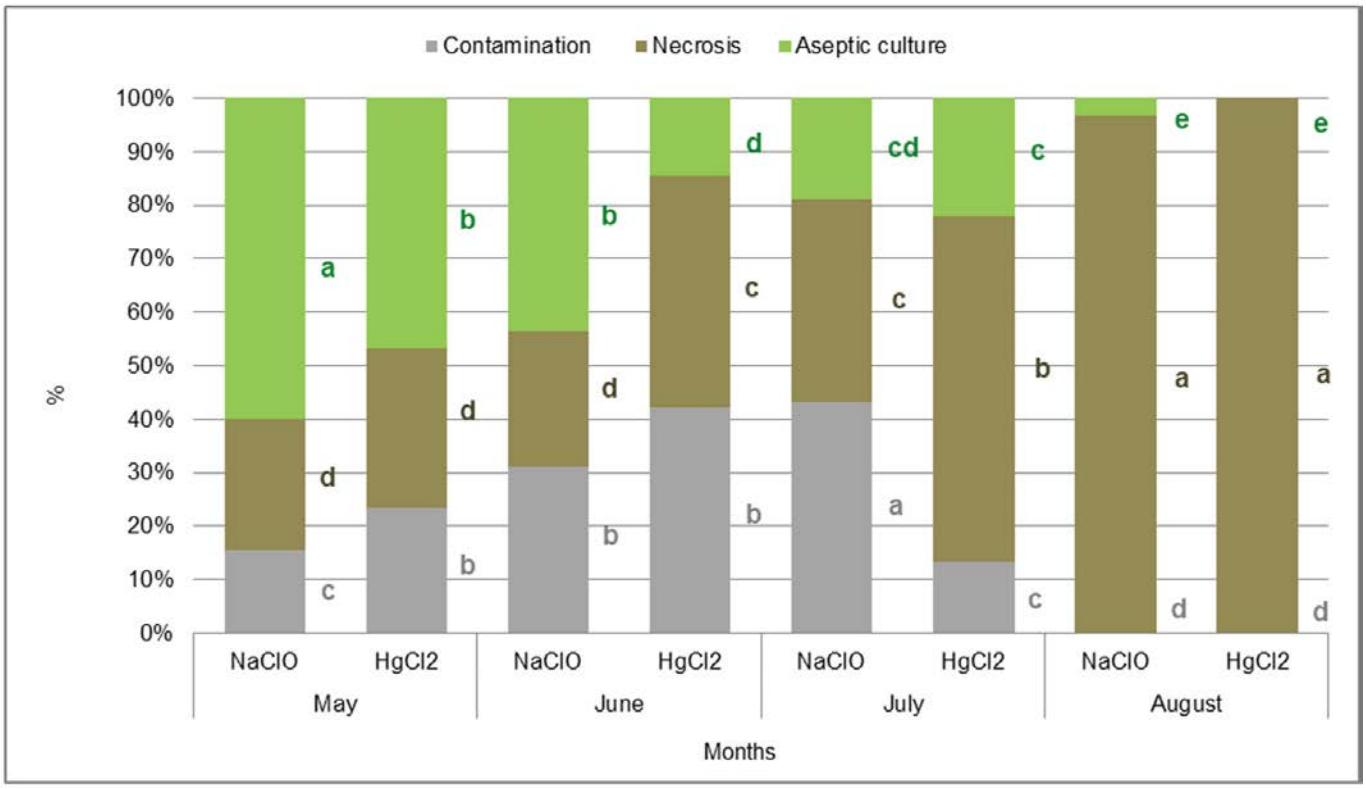

Figure 2. Contamination, necrosis and aseptic cultures in raspberry cultivar 'Meeker' (Interaction between sterilization treatments and months showed an significant impact on contamination, necrosis, and aseptic cultures. Average values for tested parameters marked with different letters are significantly different)

In contrast, for the raspberry 'Willamette', the highest percentages of contamination were recorded in May and August (48.3 and 51.7%; Table 5), while the highest percentage of explants necrosis was observed in July (49.9%). The most suitable month for establishing an aseptic culture in this cultivar was June, with 40.3% of aseptic cultures with well-developed leaf rosettes. In cultivar 'Willamette', the treatment with 0.1% $HgCl_2$ for 3 minutes resulted in the highest percentage of aseptic culture (35.4%) and the lowest infection percentage (22.6%), in contrast to the treatment with sodium hypochloride (5.6% and 55.4%, respectively).

The percentages of necrosis and aseptic culture were significantly affected by the interaction of the analyzed factors (the month of explant collection and the sterilization treatment used) in raspberry 'Willamette' (Figure 3).

Table 5. Evaluation of optimal month for establishing an aseptic culture of raspberry cultivar 'Willamette'

Factors		Contaminated cultures (%)	Explant necrosis (%)	Aseptic culture (%)
Month (A)	May	48.3^{a*}	38.9^{b}	12.8^{b}
	June	24.5^{b}	35.2^{b}	40.3^{a}
	July	30.6^{b}	49.9a	19.5^{b}
	August	51.7^{a}	33.9^{b}	14.4^{b}
Treatment (B)	II	55.4^{a}	37.0	5.6^{b}
	VI	22.6^{b}	42.0	35.4^{a}
ANOVA				
A		*	*	*
B		*	ns	*
A x B		ns	*	*

*Average values for examined parameters in the same column marked with different letters are significantly different (Duncan Multiple Range Test, $P < 0.05$)

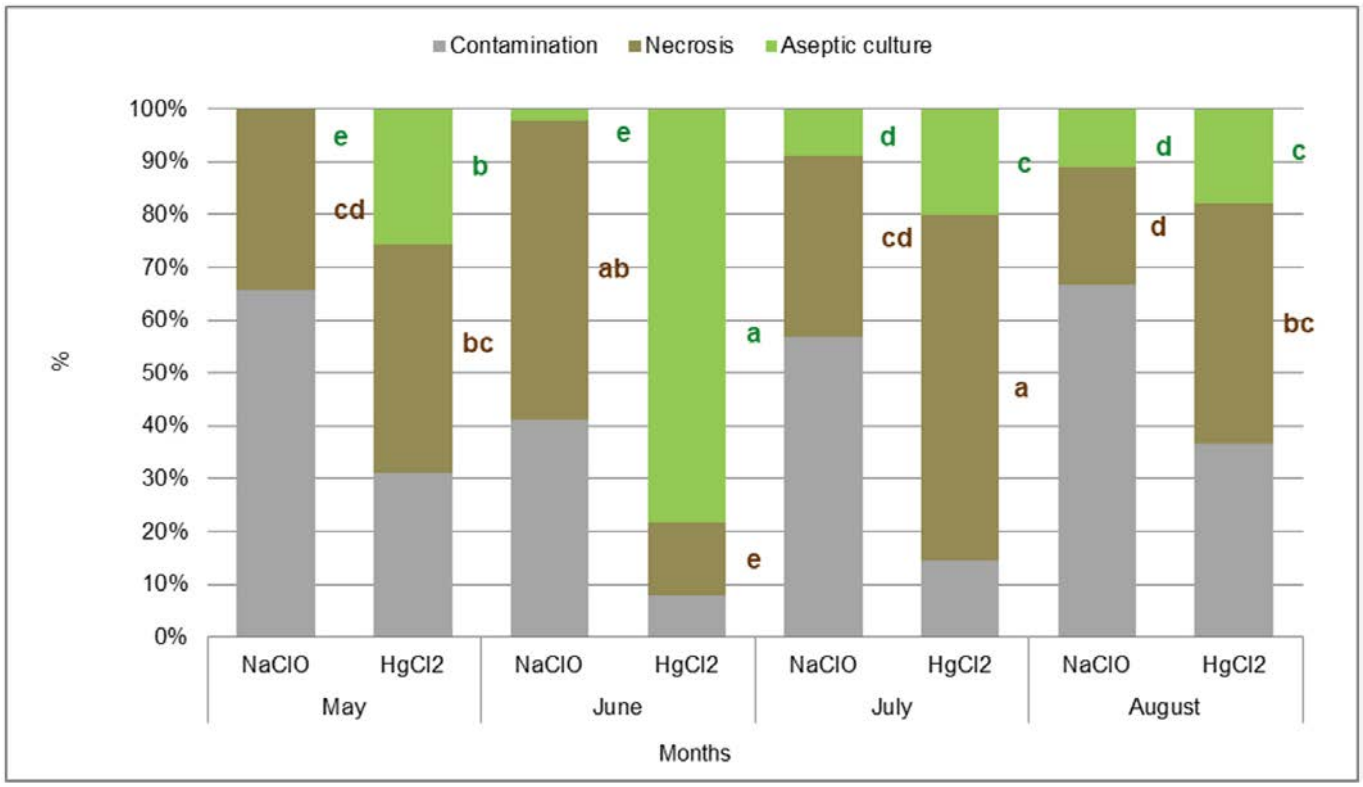

Figure 3. Contamination, necrosis and aseptic cultures in raspberry cultivar 'Willamette' (Interaction between sterilization treatments and months showed a significant impact on necrosis and aseptic cultures. Average values for tested parameters marked with different letters are significantly different, except for contamination)

When applying the surface sterilization treatment of initial explants with sodium hypochloride for 20 minutes, significantly lower values of aseptic culture were observed across all tested months compared to treatments with $HgCl_2$ for 3 minutes. It is worth noting that the highest success of establishing an aseptic culture was recorded in June, reaching 78.3% for the treatment with $HgCl_2$. June is often considered an ideal time for collecting plant material (explants) for initiating *in vitro* cultures in raspberries, as it aligns with the active growth phase for raspberry plants (Turdiyeva et al, 2023). Siwach et al. (2001) also recommended May and June as the optimal period for establishing an aseptic culture for fig. In their research, the lowest values of necrotic explants were observed in June when $HgCl_2$ was applied (13.7%), in comparison to other testing periods. During two experimental years, the application of Treatment II showed the best results for establishing an aseptic culture in the 'Meeker' cultivar, but with a high contamination rate when using $HgCl_2$. In contrast, the cultivar 'Willamette' showed higher sensitivity to bleach solution, while the application of $HgCl_2$ had the greatest impact on the percentage of establishing an aseptic culture. Research conducted by Georgieva et al. (2020) also revealed high contamination (74.8%) in cultivar 'Willamette' when using a bleach solution, which aligns with our findings, although in cultivar 'Meeker' contamination was higher than in our study (72%).

CONCLUSION

One of the most critical steps in setting up an efficient protocol for *in vitro* propagation of any genotype is the establishment of an aseptic culture. The success of this step largely depends on the physiological stage of the donor plant and genotype as well as the application of a suitable sterilization procedure. The results of this study indicate different procedures for surface sterilization in two raspberry cultivars. The best sterilization protocol for raspberry cultivar 'Meeker' included a combination of 70% EtOH for 1 minute followed by 10% NaClO for 20

minutes, while for raspberry cultivar 'Willamette', it was the treatment of 70% EtOH for 1 minute followed by 0.1% $HgCl_2$ for 3 minutes. According to the results of our study, the highest frequency of aseptic cultures and leaf rosette initiation (60%) for cultivar 'Meeker' was achieved during May, while June was found to be the most suitable month for successful (78.3%) aseptic culture establishment and leaf rosette initiation for cultivar 'Willamette'.

Based on the obtained results, our study confirmed that the same disinfection procedure cannot be used universally, especially since it is necessary to optimize sterilization protocol for different cultivars within the same species. These differences among cultivars are the result of their sensitivity to various compounds

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