Optimization of berry crop genotype regeneration systems at the *in vitro* crop initiation stage

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Abstract. The article presents data obtained during the study of the effectiveness of the initial stage of *in vitro* introduction of berry crops into culture: black currant (*Ribes nigrum* L.) and pine strawberry (*Fragaria* × *ananassa* Duch.) depending on the period of implementation and sterilizing agents used. The objects of research are varieties of black currant (Azhurnaya, Orlovskaya Serenada, Ocharovanie, Chudnoye Mgnovenye); pine strawberry (Asia, Alba, Bereginya, Darselekt, Kimberly, Klery, Marmolada Onebor, Siria, Honeoye, Florence, Frida, Tsaritsa, Urozhajnaya CGL). Introduction into culture *in vitro* was carried out in several periods characterized by different physiological state of explants: dormancy release, active growth, and retardation. Cultivation was carried out on Murashige-Skoog medium with the addition of 6-BAP (0.5 mg/l). It is noted that the survival rate of berry crop explants is determined by the physiological state of the source material, due to the period of introduction, and the genotypic characteristics of the varieties. The study of various sterilization modes of the initial plant material showed the effectiveness of such sterilizing agents as hydrogen peroxide (12.0%), merthiolate (0.01%), mercury bichloride (sulema, 0.1%), argentic nitrate (0.2%).

1 Introduction

The introduction of plants into culture *in vitro* consists in isolating plant tissues and organs, followed by cultivation on an artificial nutrient medium. At this stage, the release of plant material from sources of microbiological contamination of the nutrient medium should be carried out and reliable regeneration of plants from isolated explants should be obtained. The realization of the regenerative potential of explants depends on the influence of a combination of genetic, physiological, chemical, and physical factors: genotype, seasonality of isolation, cultivation conditions [1-18].

To free the sources of berry crop explants from possible infection at the initiation stage, different authors offer various options for sterilizing agents (solutions of merthiolate, sulema, hydrogen peroxide, sodium lauryl sulfate, fungicide oxychome, etc.) and schemes for their use [2-11].

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Successful introduction to culture is also determined by the terms of obtaining explants. Tissues and organs isolated at the time of plant vegetation have a higher morphogenetic potential compared to tissues taken as an explant during deep and forced dormancy [7-11]. Nevertheless, N.V. Kukharchik considers February-the beginning of April to be the best time for introduction into culture in vitro – the phase of vegetative kidney release from the dormant period [8].

The purpose of these studies was to study the effectiveness of the initial stage of introduction into culture *in vitro* of various berry crops (black currant and pine strawberry), depending on the period of introduction, appropriate sterilization regimes, and genotypic characteristics of varieties.

2 Materials and methods of research

The studies were conducted in 2016-2021 according to generally accepted methods [1, 2, 8, 12].

The objects of the study were berry crops:

– black currant: varieties Azhurnaya, Orlovskaya Serenada, Ocharovanie, Chudnoye Mgnovenye;

– pine strawberry garden: varieties Asia, Alba, Bereginya, Darselekt, Kimberly, Klery, Marmolada Onebor, Siria, Honeoye, Florence, Frida, Tsaritsa, Urozhajnaya CGL.

The introduction of black currant into the culture *in vitro* was carried out in three periods characterized by different physiological state of explants. In the spring period of dormancy release (mid-March), the closed buds of annual lignified shoots served as the starting material. To stimulate the process of dormancy release, the shoots were placed in a vessel with water and kept at a temperature of +22...+24°C until the appearance of a green cone. In the summer period (June), which is characterized by active growth, the growing green shoots served as a source of buds. In the autumn period (mid-September) there is a growth fading and a transition to a dormancy state, during this period the source material was the closed buds of annual lignified shoots.

The introduction of pine strawberry into the culture *in vitro* was carried out in three periods: growth beginning (February), active growth (June), and growth fading (August). Since strawberry does not have a pronounced dormancy period, winter storage of unrooted rosettes is possible and their use for introduction into culture *in vitro* with the growth onset. The rosettes were harvested in October, cleaned of covering leaves and roots and stored in plastic bags in the refrigerator at a temperature of +1...+2°C. In the summer period (June, August), the starting material was the apical buds of stolons, young or already formed rosettes harvested before introduction into culture *in vitro*.

Sterilization of plant material was carried out using the following sterilization schemes (Table 1).

Table 1. Sterilization schemes of initial plant material

No.	Sterilization method	Processing time
Black currant		
1	Running tap water 70.0% ethyl alcohol Sterile distilled water Mercury bichloride (0.1%) Sterile distilled water (3 times)	40 min. 10 sec. 10 min. 10 min. for 10 min.
2	Running tap water 70.0% ethyl alcohol	40 min. 10 sec.

	Sterile distilled water Merthiolate (0.1%) Sterile distilled water (3 times)	10 min. 10 min. for 10 min.
Pine strawberry		
3	Detergent "Fairy" Running tap water 70.0% ethyl alcohol Sterile distilled water Hydrogen peroxide (12.0%) Sterile distilled water (3 times)	1 min. 40 min. 10 sec. 10 min. 5 min. for 10 min.
4	Detergent "Fairy" Running tap water 70.0% ethyl alcohol Sterile distilled water Merthiolate (0.01%) Sterile distilled water (3 times)	1 min. 40 min. 10 sec. 10 min. 10 min. for 10 min.
5	Detergent "Fairy" Running tap water Hydrogen peroxide (3.0%) Sterile distilled water 70.0% ethyl alcohol Sterile distilled water Mercury bichloride (0.1%) Sterile distilled water (3 times)	1 min. 40 min. 1 min. 1 min. 10 sec. 10 min. 10 min. for 10 min.
6	Detergent "Fairy" Running tap water Hydrogen peroxide (3.0%) Sterile distilled water 70.0% ethyl alcohol Sterile distilled water Silver Nitrate (0.2%) Sterile distilled water (3 times)	1 min. 40 min. 1 min. 1 min. 10 sec. 10 min. 2 min. for 10 min.

The viability indicators were the proportion of viable explants (live uninfected explants suitable for further cultivation), the proportion of dead explants (necrosis), including uninfected dead meristems and the proportion of infected meristems (contamination), including infected dead and live meristems.

A modified Murashige-Skoog medium [19] with the addition of cytokinin 6-BAP (0.5 mg/l) was used to cultivate the meristematic tops obtained from the buds.

Statistical processing was carried out using Microsoft Excel 2016 computer program. The degree of influence of the introduction period and sterilizing agents on the effectiveness of culture initiation *in vitro* was evaluated using the variation coefficient of the indicator (CV). The value of CV ≤ 10% is a weak variability of the indicator, at 10% ≤ CV≤ 20% – average, and CV more than 20% – high.

To assess the significance level of the indicator, the Student's criterion (T) was used with the significance of the indicator P≤0.05.

3 Results and their discussion

3.1 Black currant

Factors such as the period of isolation of explants, genotypic features of varieties and the scheme of sterilization of explants have a significant impact on the effectiveness of black currant introduction into culture *in vitro* [4, 7, 8, 9, 14-16].

In our study, when introduced into culture *in vitro* in the spring, viability indicators, in particular the proportion of dead and infected meristems, are characterized by high variability, since varietal characteristics have a significant impact. The physiological state of the initial buds (the beginning of dormancy release, the degree of openness of the meristems) and the differentiation of the buds by flower type cause an increase in the proportion of dead explants under the action of a sterilizing agent and due to necrosis of the preserved rudimentary inflorescences. The varieties Azhurnaya, Orlovskaya Serenada, and Chudnoye Mgnoveniye react most sharply to the effect of external factors at the stage of culture initiation *in vitro*.

Explants isolated during the summer period (active vegetation period) are characterized by a higher and stable survival rate, since the meristems of the apical buds of green shoots are fully formed, have significant growth potential, and are free of pathogens.

The indicators of explant viability in the autumn period indicate the influence of the genotypic features of the varieties, manifested in the morphological features of the buds. The initial state of plants during the growing season, the timing of the onset and duration of growth fading, the degree of development of meristems determine the unstable development of meristematic tops. In addition, the formation of buds in the flower buds of the next year leads to an increase in the proportion of dead explants, similar to the spring period.

The analysis of the effectiveness of the use of sterilizing agents showed that the most effective and less toxic for explants of all varieties was mercury bichloride (0.1%) compared with merthiolate (0.01%). At the same time, it was noted that the viability indicators also correlate with the period of introduction and varietal characteristics. Thus, when treated with mercury bichloride (0.1%) in the spring and summer periods, the proportion of infected explants does not exceed 4%, which is explained by the openness of the meristems and their availability for the sterilizing agent.

Fig. 1. The source material (apical buds of green shoots) of the "Azhurnaya" variety after sterilization with mercury bichloride (0.1%) during the summer period of introduction

The number of infected meristems in the autumn period increases slightly (up to 9%), which is due to the morphological features of the buds of various varieties. The tightly closed casing scales of the buds of the Azhurnaya, Ocharovaniye, and Chudnoye Mgnoveniye varieties prevent the penetration of the sterilizing agent, and the small meristems located along the edge are more susceptible to the traumatic effects of the sterilizer.

When treated with merthiolate (0.01%) in the summer period, the proportion of dead explants is high, while this indicator varies significantly by variety (from 13% in the Azhurnaya variety to 57% in the Orlovskaya Serenada variety). This indicates the high toxicity of this substance for open active meristematic tissues. In the autumn period of introduction, the number of dead explants is lower and does not exceed 17%, which is due to the protection of the casing bud scales.

The results of research are presented in Table 2.

Table 2. Viability of black currant explants depending on the introduction period, sterilizing agent used, and varietal characteristics, 2018-2021.

Varieties	**Viability indicators, %**	**Period of introduction into culture**			**CV**
		spring	**summer**	**autumn**	
Sterilizer: mercury bichloride (0.1%)					
Azhurnaya	Percentage of viable explants	73	90	81	*8.5%*
	Necrosis	24	8	17	*40.1%*
	Contamination	3	3	2	*17.7%*
Orlovskaya Serenada	Percentage of viable explants	74	85	90	*8.1%*
	Necrosis	25	12	7	*51.7%*
	Contamination	2	4	4	*28.3%*
Ocharovaniye	Percentage of viable explants	66	83	72	*9.6%*
	Necrosis	34	17	19	*32.5%*
	Contamination	0	1	8	*118.6%*
Chudnoye Mgnoveniye	Percentage of viable explants	48	74	63	*17.3%*
	Necrosis	48	22	28	*34.0%*
	Contamination	3	4	9	*49.2%*
Student's criterion, T (for indicator 1)*		$T_{spring-summer}=2.6$	$T_{summer-autumn}=1.0$		$T_{spring-autumn}=1.3$
Student's criterion, T (for indicator 2)**		$T_{spring-summer}=2.8$	$T_{summer-autumn}=2.1$		$T_{spring-autumn}=0.6$
Student's criterion, T (for indicator 3)***		$T_{spring-summer}=1.0$	$T_{summer-autumn}=1.5$		$T_{spring-autumn}=2.1$
Coefficient of variation (CV) (for indicator 1)		*16.0%*	*7.0%*		*13.2%*
Coefficient of variation (CV) (for indicator 2)		*29.4%*	*35.7%*		*42.0%*
Coefficient of variation (CV) (for indicator 3)		*61.2%*	*40.8%*		*49.8%*
Sterilizer: merthiolate (0.01%)					
Azhurnaya	Percentage of viable explants	—	85	83	*1.2%*
	Necrosis	—	13	13	*0.0%*
	Contamination	—	2	4	*33.3%*
Orlovskaya Serenada	Percentage of viable explants	—	36	94	*44.6%*
	Necrosis	—	57	3	*90.0%*
	Contamination	—	7	2	*55.6%*

Ocharovaniye	Percentage of viable explants	—	81	76	*3.2%*
	Necrosis	—	15	17	*6.3%*
	Contamination	—	4	7	*27.3%*
Chudnoye Mgnoveniye	Percentage of viable explants	—	75	86	*6.8%*
	Necrosis	—	22	5	*63.0%*
	Contamination	—	3	9	*50.0%*
Student's criterion, T (for indicator 1)		—	$T_{summer-autumn}$=1.3		
Student's criterion, T (for indicator 2)		—	$T_{summer-autumn}$=1.6		
Student's criterion, T (for indicator 3)		—	$T_{summer-autumn}$=0.8		
Coefficient of variation (CV) (for indicator 1)		—	*28.2%*		*7.6%*
Coefficient of variation (CV) (for indicator 2)		—	*66.5%*		*60.2%*
Coefficient of variation (CV) (for indicator 3)		—	*46.8%*		*49.0%*

Note 1: *Indicator 1 – proportion of viable explants; ** Indicator 2 – necrosis, *** Indicator 3 – contamination.
Note 2: the table shows the average values of explant viability indicators for the specified research period

3.2 Pine strawberry

The success of *in vitro* initiation of pine strawberry culture depends on the complex impact of factors such as the time of introduction, the physiological state of the explant, the type of sterilizing agent, and the sterilization scheme. For most berry crops, the best period for introduction into culture *in vitro* is the phase of plant dormancy release or the beginning of active growth [8, 12, 13]. Pine strawberry, unlike other plants, do not have a pronounced dormancy period, which allowed the introduction of meristems into the culture *in vitro* in February after winter storage of rosettes at low positive temperatures. The conducted studies have shown that during this period the apical meristems showed the greatest regenerative ability and the stabilization of the culture occurred faster. The proportion of viable explants in all the studied varieties was high and amounted to 65...94%. The varietal specificity was manifested most sharply when introduced into the phase of active growth, the survival rate of explants was 52...93%. During the period of growth fading (August), the genotypic features of the varieties were manifested to the least extent while maintaining a significant proportion of viable explants (61...86%).

The results of the conducted studies are shown in Table 3.

Table 3. Dependence of viability of strawberry explants on the period of introduction, 2018-2021, % (sterilizer – mercury bichloride (0.1%))

Variety	Period of introduction into culture			CV
	February (growth beginning)	June (active growth)	August (growth fading)	
Asia	84	77	77	*4.0%*
Alba	87	92	74	*9.0%*
Bereginya	82	93	69	*12.0%*
Darselect	69	87	79	*10.0%*
Kimberly	77	72	77	*3.0%*
Klery	78	52	61	*17.0%*
Marmelada	65	84	86	*12.0%*
Syria	94	90	77	*8.0%*

Urozhaynaya CGL	82	88	72	*8.0%*
Florence	87	62	76	*14.0%*
Honeoye	83	68	85	*10.0%*
Tsaritsa	84	87	79	*4.0%*
Student's criterion, T	$T_{February-June}$=0.4		$T_{June-August}$=0.8	$T_{February-August}$=1.7
Variation coefficient (CV)	*9.4%*	*15.9%*	*8.5%*	

Note: the table shows the average values of explant viability indicator for the specified research period

Merthiolate (0.01%), mercury bichloride (0.1%), hydrogen peroxide (12.0%) were used to free the source material of strawberry from saprophytic microflora and silver nitrate (0.2%). The best sterilizing effect was obtained by stepwise sterilization of the starting material using mercury bichloride (0.1%) as the main sterilizing agents (Table 1, method 5) and silver nitrate (0.2%) (Table 1, method 6). At the same time, the degree of infection and tissue damage did not exceed 5-10%. Hydrogen peroxide had the least sterilizing effect (12.0%) and merthiolate (0.01%) (Table 1, methods 3, 4), reducing the viability of plant material.

The research results of the effectiveness of the use of sterilizers in the winter period of introduction are shown in Figure 2.

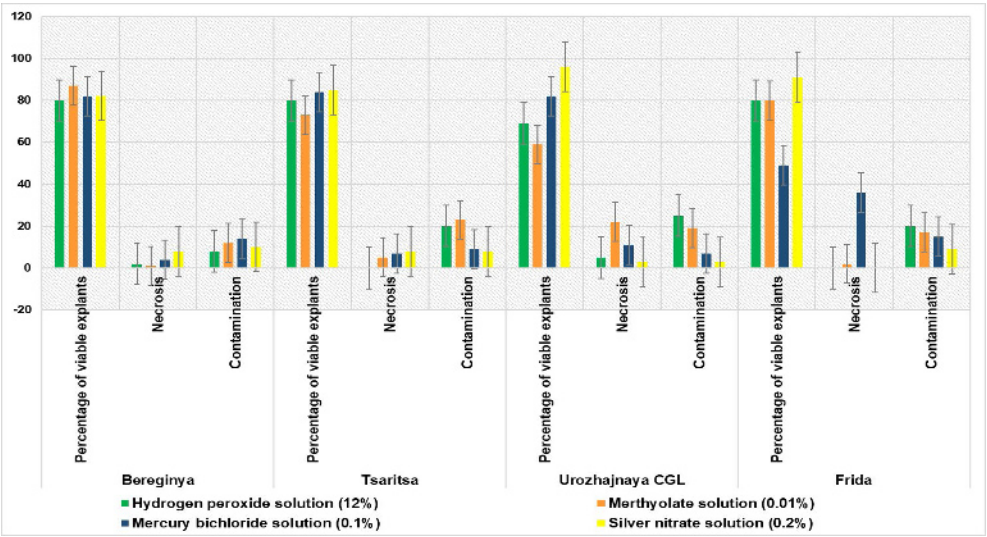

Fig. 2. The effectiveness of the use of sterilizers on strawberry varieties during winter introduction, 2016-2021, %

When introducing strawberry explants into the culture *in vitro*, there is a negative effect of phenol oxidation products on the wound tissues of explants, which inhibits growth processes. In this regard, the initial sterile material before separation is contained in Petri dishes in a sterile solution of an antioxidant – ascorbic acid (0.3%). Complete elimination of oxidation is achieved by transplanting explants to a fresh nutrient medium after 16...18 hours.

4 Conclusions

When black currant is introduced into culture *in vitro*, the most significant influence is exerted by the timing of isolation of explants and genotypic features of varieties. Explants isolated during the active vegetation period (summer) are characterized by a higher and stable survival rate. When cultivating explants in the spring and autumn periods, the physiological state of explants and their survival rate are influenced by the specific reaction of varieties to the timing of dormancy release and the duration of the growth fading period. The most effective sterilizing agent for black currant explants is mercury bichloride (0.1%).

The most favorable period for the introduction of pine strawberry into the culture *in vitro* is the period of dormancy release (February), since the apical meristems showed the greatest regenerative ability. The best sterilizing effect of the source material was achieved by using mercury bichloride (0.1%) and silver nitrate (0.2%).

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