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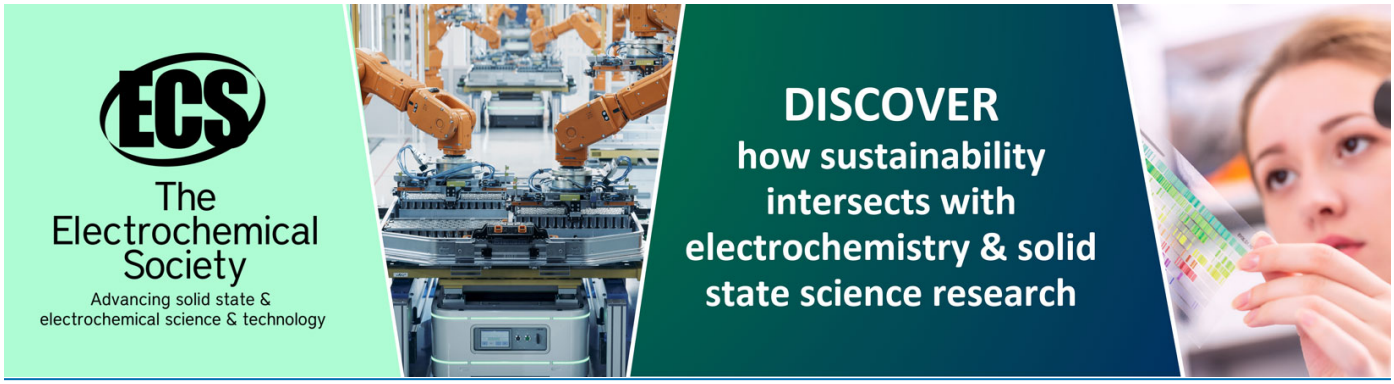

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Phytopathogenic mycobiota of the Far Eastern species of the genus *Aristolochia* L. in the culture *in vitro*

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Abstract. Microclonal reproduction of plants of the Far Eastern species of the genus Aristolochia L on explants revealed the micromycetes of various systematic groups. In microclonal propagation, the authors used various methods of sterilization of the starting material with diacid and sublime. The total period of manifestation of the pathogenic properties of micromycetes on the explants of species of the genus Aristolochia L. was 24 days *in vitr*o.

1. Introduction

The family Aristolochiaceae (Aristolochiaceae Juss.) has 7 genera and more than 650 species of woody lianas and perennials, distributed mainly in the tropics and subtropics of both hemispheres. Some members of the genus Aristolochia L. attributed to the relics of the Tertiary period [14]. The genus Aristolochia L. usually occurs in tropical latitudes; in Russia, the genus Aristolochia L. is represented by five species, of which two grow in the Far East: Aristolochia manshuriensis Kom., A. contorta Bunge, and one A. clematitis L.

A. manshuriensis Kom. and A. contorta Bunge grow in the south of Primorsky Krai, outside of Russia, their range covers some provinces of North-East China, A. contorta grows on parts of Japan and the Korean Peninsula [10]. Natural populations of two representatives of the *kirkazon* genus – Aristolochia manshuriensis and A. contorta – are highly fragmented and scarce [8], which makes them particularly vulnerable, these species are rare in the flora of the Far East, listed in the Red Book of Primorsky Krai with the status of endangered (EN) (A. manshuriensis) and (VU) vulnerable (A. contorta) [6].

The weak seed productivity and absence of natural seed propagation agents among representatives of the Far Eastern flora lead to a permanent reduction in the number, including *kirkazon* (Aristolochia manshuriensis). The presence of unique medicinal properties in species of the genus leads to the extermination of rare individuals as a medicinal raw material.

The restoration of natural populations of rare and endangered plant species by the method of clonal micropropagation is a promising direction of biotechnology. Biological features of the growth and development of *kirkazon* is associated with the hindered vegetative and seed reproduction, a high degree of infection with fungal and bacterial diseases, and a single occurrence of individuals at reproductive age [5]. In this case, the condition for obtaining high-quality raw materials for the cultivation of regenerated plants is the use of a sterile culture obtained by the method of microclonal propagation.

The micromycetes on the vegetative organs (source material) causing the death of the explant can be a serious reason preventing the introduction of plants into the culture *in vitro*.

The issues of the phytopathogenic state of the species of the *kirkazon* genus in natural populations have been studied in foreign and domestic literature. Mycobiota of the Far Eastern species of *kirkazon*, obtained by the method of microclonal reproduction, has not been studied to date.

Studies of the species diversity of parasitic fungi on explants of plants of the genus Aristolochia L. will allow optimizing methods for sterilizing the source material introduced into an *in vitro* culture.

In connection with the foregoing, the purpose of this work is to study the phytopathogenic microbiota on explants of the Far Eastern species of the genus Aristolochia L. *in vitro* and to optimize the method of sterilization of the starting material.

To solve it, the following tasks were set:

1. To identify species diversity and characteristics of the development of phytopathogenic micromycetes on explants of Far Eastern representatives of the genus Aristolochia L. in vitro.
2. To optimize the methods of sterilization of the source material introduced into the culture *in vitro*.

2. Materials and Methods

Objects of study for introduction into culture in vitro are the species of the *kirkazon* genus: Aristolochia manshuriensis, A. contorta Bunge, and A. clematitis L.

The plant material is collected in the collection of the arboretum belonging to the Branch of the Federal Scientific Center for Terrestrial Biota of East Asia of the Far Eastern Branch of the Russian Academy of Sciences at the Gornotaezhny Station named after V.L. Komarova (hereinafter referred to as "Gornotaezhny Station)".

Young shoots of the virginal age of the current year of development with one or two axillary buds (explants), which were taken in June, were used as the starting material. Studies were performed on 20 explants of each plant species in triplicate.

During the experiment, the proportion of explants that were affected by the fungi was estimated.

Sterilization of the starting material (microcurrents) was carried out in two ways. In the first case, the explants were sterilized by the standard technique, which consisted in the sequential processing of a soap-alkaline solution (15 min) and a 0.1% solution of the diacid (4 min) with 3-fold washing with sterile distilled water [5;1]. In the second, a 0.2% mercury chloride solution was used to sterilize the source material (micro-cuttings) II ($HgCl_2$) (mercuric chloride) within 4 minutes, with 3-fold washing in a sterile distilled water.

Under boxing conditions (BAVnp-01 "Laminar-S.," 1.2), our explants were placed vertically in the previously prepared sterile nutrient media. Preliminary preparation of all instruments was carried out in a glossy sterilizer (S-01). The working surface was disinfected with ethyl alcohol (70%).

Morphometric measurements of the samples were carried out in an aqueous solution using a Nikon Eclipse E200 microscope at 80–400X, with 1500X magnification. Microscopic examination was performed according to generally accepted botanical techniques [15], [3]. Latin names of mushrooms and plants, and abbreviations of authors' names are in accordance with the open database "Index Fungorum, 2018" [16].

After sterilization, the cuttings were placed vertically in the nutrient media prepared on the basis of macro and micro salts in MS and WPM with the addition of indole acetic acid (IAA) at a concentration of 1.0 mg / l and 4.0 mg / l and 2ip - 8 mg / l [4].

The nutrient media were sterilized by autoclaving at 0.8 atm for 20 minutes. Test tubes with a diameter of 10 mm and a height of 15 cm were used as culture vessels for growing microshoots.

Explants at all stages of the experiment were cultured at a temperature of +24 ºC, a 16-hour photoperiod (16/8), illuminated with white fluorescent lamps with an intensity of 4 thousand lux. and 60% relative humidity [5].

3. Results

As a result of our research, taking into account the conditions of sterilization of explosives, *in vitro* cultivation of the species with the genus Aristolochia L., the micromycetes of different systematic groups were found (Table 1).

Table 1. The causative agents of fungal phytopathogenic infections during clonal micropropagation of species with the genus Aristolochia L.

Species	Phytopathogenic micromycetes	
	Sterilization diacidom	**Sterilization $HgCl_2$**
A. manshuriensis Kom.	Alternaria alternata (Fr.) Keissl., Botrytis cinerea Pers., Fusarium oxysporum Schlecht., Rhizopus stolonifer (Ehrenb.), Mucor hiemalis Wehmer., Trichothecium roseum (Pers.) Link.	Fusarium oxysporum Schlecht., Fusarium oxysporum Schlecht., Rhizopus stolonifer (Ehrenb.), Trichothecium roseum (Pers.) Link.
A. contorta Bunge.	Alternaria alternata (Fr.) Keissl., Botrytis cinerea Pers., Mucor hiemalis Wehmer.	Not identified
A. clematitis L.	Botrytis cinerea Pers., Mucor hiemalis Wehmer.	Not identified

Observations have shown that during sterilization by diacid, explants of A. manshuriensis Kom. are more susceptible to infection. At the same time, the A. contorta Bunge, which occupies a transitional position between woody and grassy species of the genus, is struck by a significantly smaller number of pathogenic mycobiota species. In the herbaceous species A. clematitis L., explants *in vitro* are susceptible to infection with only two types of mold fungi.

When using the sublimate solution, we observed the presence of only 3 types of micromycetes on A. manshuriensis explants *in vitro*: Fusarium oxysporum Schlecht., Rhizopus stolonifer (Ehrenb.), Trichothecium roseum (Pers.) Link. All these types of micro-cuttings struck the A. manshuriensis Kom.

According to literary data in grassy species and species that are transitional between grassy and ligneous plants, the main phase of the mass detection of infected explants occurs at the end of the third week of cultivation. It has been established that the main condition preceding the onset of development of fungal phytopathogenic microorganisms during microclonal propagation of plants *in vitro* is increased humidity, an insufficiently effective method of sterilizing primary explants and feeding habits of phytopathogenic micromycetes.

The total period of manifestation of phytopathogenic microflora on non-sterile micrograins and micro-shoots of species of the genus Aristolochia L. in vitro was 24 days from the initial landing.

The first manifestations of visible signs of fungal infection on the explants were observed on days 5-7 of cultivation (Fig. 1).

Figure 1. Fungal infection in the cultivation of Aristolochia manshuriensis using sterilization by diacid (on the right, the explants affected – Mucor hiemalis; Fusarium oxysporum on the left).

In each test tube with the explants, only 1 type of micromycetes was developed. After 3 days of cultivation, micromycetes appeared in 100% of the explants, the explants of all three species completely blackened and died. In the first week of cultivation, a fungal infection was observed in

approximately 30% of A. manshuriensis and A. contorta explants, this figure was 2 times lower for A. clematitis. The proportion of infected explants A. contorta and A. manshuriensis for 10 days of cultivation increased in 1.3 times. A. alternata (28% of the explants out of 41.7%) was noted most often in test tubes on explants of A. contorta, 28.7% of B. cinerea and M. hiemalis were recorded. The explants for A. clematitis were M. hiemalis (10.8% of 15%) and B. cinerea (4%).

In the course of the research, it is found that the highest rate of activity of pathogenic microorganisms was observed from the 2nd week from the moment of planting until the middle of the 3rd, during these periods the percentage of infection of the microdrops sharply increased, and was 2 times higher than in the 1st week of cultivation (Table 2).

Table 2. Infectiousness of explants of Aristolochia L. species when using diacine sterilization after 24 days of cultivation.

Species	The proportion of affected explants, % by day						
	6	**7**	**10**	**14**	**17**	**21**	**24**
A. manshuriensis Kom.	28.9	30	37.8	67.1	67.5	93.3	100
A. contorta Bunge	28.3	27.5	38.3	41.7	41.2	96.7	100
A. clematitis L.	5,6	11.1	15	20	22	38.3	40

After 3 weeks of cultivation, the micromycetes appeared on almost all explants of A. contorta and A. manshuriensis, while about 60% of A. clematitis explants showed no signs of infection. After two weeks of cultivation (before the end of the experiment), B. cinerea was most often observed on the explants of A. contorta and A. manshuriensis, without obvious signs of fungal infection on the explants of A. clematitis M. hiemalis. Since phytopathogenic micromycetes developed on all explants in the absence of exchanges of pathogens and the inflow of propagules of phytopathogenic fungi from the outside, it can be concluded that the infection of the studied A. contorta and A. manshuriensis samples was initially 100%.

A slightly different picture of infection of the studied species of the genus *kirkazon* when using the sterilization of sublimate (Table 3).

Table 3. Infection of explants of Aristolochia L. species when using sterile sterile sterilization after 24 days of cultivation.

Species	The proportion of affected explants, % by day						
	6	**7**	**10**	**14**	**17**	**21**	**24**
A. manshuriensis Kom.	8.0	12.0	12.0	26.0	29.0	37.0	49.0
A. contorta Bunge	-	3.1	3.1	4.0	4.8	6.3	6.3
A. clematitis L.	-	-	1.3	1.7	2.1	2.4	2.4

The use of a more concentrated solution of sublimate as a sterilizer led to a significant reduction in the number of identified pathogenic micromycetes on *in vitro* kirkazon explants. In the case of A. manshuriensis microcurrents, the amount of cultivated material infected by internal infection averaged 50%, the similar indicators did not exceed 5-10% in the grassy species.

The main symptoms of diseases caused by pathogens of pathogenic fungi are: the appearance of dark brown or olive-black raids, consisting mainly of conidiophores and conidia of the fungus (Alternaria alternata (Fr.) Keissl.); the formation of a thick fluffy dark gray patina sprinkled with light gray flour (Botrytis cinerea Pers); a lesion in the form of pink (with shades of soft pink to crimson color) mold; and it was accompanied by the rotting of internal tissues (Fusarium oxysporum Schlecht.) sporangia of the fungus as black heads (Rhizopus stolonifer (Ehrenb), the formation of a fluffy white plaque which darkens with (Mucor hiemalis Wehmer) over time [2], [7], [9], [11], [12].

It is known that the antifungal effect is shown for species of the genus Aristolochia. Therefore, the extracts of A. debilis, A. bracteolata Linn, and A. ringens suppressed the development of Botrytis cinerea [18]; the extracts of A. indica and A. debilis affected the development of F. oxysporum [19], the extracts of A. bracteolata influenced R. stolonifer [17]; while the extracts of A. contorta affected M. hiemalis et al. [13].

According to the above, we assume that the micromycetes found as a result of the experiment are not brought outside (with the initial material), but are inside the explants tissues.

According to the data obtained, the method used for diacine sterilization of primary explants during the cultivation of A. manshuriensis *in vitro* promotes the development of pathogenic micromycetes. In some cases, a longer period of treatment with diacid leads to damage to both the outer integumentary tissues of the plant's microcurrents and the tissues on the cutting section, which entails the partial or complete die-off of the damaged plant tissues.

4. Conclusion

The species diversity of parasitic fungi was carried out on the explants of plants of Far Eastern species of the genus Aristolochia L., which manifest themselves in microclonal reproduction in vitro.

According to the obtained results, the phytopathogenic micromycetes are represented by two sections – Zygomycota and Ascomycota, 6 genera and 6 species (Alternaria alternata (Fr.) Keissl., Botrytis cinerea Pers., Fusarium oxysporum Schlecht., Rhizopus stolonifer (Ehrenb.), Mucor hiemalis Wehmer., Trichothecium roseum (Pers.) Link.

For the plants of A. manshuriensis, a description of phytopathogenic species of fungi is given for the first time. When cultivating microcurrents and Aristolochia manshuriensis microshoots, the need to improve the method of sterilization of primary explants is shown, which is due to an increase in infection of micrografts (67.5-100%) after treatment with a diacid solution.

Among those identified in this method of sterilization, the most numerous was Fusarium oxysporum Schlecht. They affected from 15 to 35% of A. manshuriensis microcurrents. The share of Fusarium oxysporum and Rhizopus stolonifer accounted for no more than 5-15% of the total number of microcurrents. The period of manifestation of visible signs of infection of the explants was approximately 23-26 days. The explants of A. manshuriensis, which were not subjected to the manifestation of infection during this period, formed shoots and partial thickening of the epithelial tissues at the base of the microcurrent. For A. clematitis and A. contorta microchips, this sterilization method is the most acceptable, since in this case the number of explants with obvious signs of damage by pathogenic microbiota does not exceed 5-7%.

The use of biotechnological methods of reproduction contributes to the conservation and sustainable reproduction of rare and endemic species *in vitro*. The obtained data on the phytopathogenic mycobiota will allow optimizing the microclonal propagation methods of Aristolochia manshuriensis and obtaining a sufficient amount of planting material in order to restore the number of natural populations.

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