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Obtaining sterile *Calligonum aphyllum* material for in vitro culture preservation

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Abstract. The comparative evaluation's results on the efficiency of surface treatment of *Calligonum aphyllum* (Pall.) Guerke explants, a perspective plant species in the restoration of degraded lands, are presented. Sterilizers «Lysoformin 3000», silver nitrate, hydrogen peroxide, and «Belizna-econom» are used at the introduction stage of the plants in vitro. As a result, 2 of 11 effective sterilization modes were found when *C. aphyllum* was introduced into the culture in vitro: No. 10 (hydrogen peroxide solution 10% v/v, 5 min) and No. 11 (hydrogen peroxide solution 6% v/v, 15 min). These modes had the highest rates of sterile and regenerative explants. The microflora was also isolated from the studied explant samples. Determined the preparation «Lysoformin 3000» effectively acts on all isolated microorganisms. «Belizna-econom» and hydrogen peroxide (concentrations of 10% v/v) do not act on 80% of isolated bacterial strains. Shown the silver nitrate solution was not sensitive in 100% of cases. The obtained information can be used to draw up a complete protocol for *C. aphyllum* explant sterilization at the introduction stage in vitro.

1 Introduction

The problem of desertification in arid, subarid, and subhumid zones is an actual problem of national importance in the Russian Federation. The current state of the land in this area indicates a decrease in the resistance of existing ecosystems to degradation due to the trend of increasing aridity in the climate. The dynamics' analyses of these processes using geoinformation technologies and aerospace data in the North-Western Caspian by researchers [1] showed the area of degraded and deserted lands is growing due to increased pasture load.

Today, more than 40 million hectares of natural pastures, which are the forage base for raising sheep, horses, and saigas, are located in the arid regions of Russia. Irrational use has led to the degradation of pastures and the disappearance of valuable species of fodder plants. In order to restore biodiversity and increase the productivity of ecosystems changed under the influence of anthropogenic factors, V. M. Kosolapov and Z. Sh. Shamsutdinov bred 12 new varieties of arid fodder plants (included in the National Register of Breeding Achievements) [2]. They are widely used in arid regions of Russia (the Astrakhan and

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Volgograd regions, the Republic of Kalmykia, and the flat regions of Dagestan). Complexes of reclamation measures have been developed for various categories of agricultural lands for increasing the natural resource potential of arid ecosystems and aimed at preventing desertification of territories, reducing natural and anthropogenic salinity, alkalinity tolerance, and the risk of developing other negative processes [3].

One of the promising crops used for pasture restoration is Juzgun leafless, or *Calligonum aphyllum* (Pall.) Guerke. It is a shrub up to 3 m high from the family *Polygonaceae* Juss. It has a tap-root, less often a rhizome-tap-root system, of 3–7 (very rarely up to 87) partial bushes, with branches broken-tortuous at an angle of 60–80°, with red-brown bark of old branches, and it propagates well vegetatively [4]. The distribution area is the Caspian lowland, including the republics of Dagestan and Kalmykia, Kazakhstan. It grows well in a typical sandy desert on small-hilly, fixed and semi-fixed sands at the foot of dunes, sandy ridges. It is used in the national economy as a phytomeliorant [3, 5]. Researchers at the Aral Sea experimental station of VIR in the desert zone *C. aphyllum* along with *Haloxylon aphyllum* (Minkw.) Iljin. and *Ceratoides eversmanniana* were identified as the most drought tolerant among 5,000 forage crop samples. They are used with great success in creating pasture protection belts and as sand binders [6].

Currently, new varieties based on *C. aphyllum* are also emerging, for example, varieties with such valuable properties as drought resistance and high winter hardiness [5, 7]. Also, in plants of the genus *Calligonum* L., the presence of such biologically active substances as flavonoids, tannins, alkaloids, proteins, and organic acids was found. Yktiyarov A. A. and Bayduysenova A. U. discovered antimicrobial activity in *C. aphyllum* [8, 9].

An assessment of the nutritional value and biochemical composition of *C. aphyllum* leafless showed that it has significant energy resources, its biomass is a good source of increasing the forage capacity of pastures in Dagestan [10].

All of the properties listed above make *C. aphyllum* a valuable object for use in combating desertification processes and restoring lands degraded as a result of grazing pressure in arid regions. For effective reproduction in artificial conditions, the use of biotechnological methods and techniques is relevant. The use of micropropagation methods will contribute to the conservation of C. aphyllum biodiversity with its unique properties and the use of degraded areas in restoration activities.

The purpose of the research is to study the efficiency of various sterilizers when introduced into in vitro culture and to determine their effect on microorganisms isolated from *Calligonum aphyllum* explants to obtain aseptic planting material.

2 Materials and methods

The studies were carried out in 2022 in the Laboratory of Biotechnologies of the Federal Scientific Center of Agroecology of the Russian Academy of Sciences. *C. aphyllum* microcuttings with 1-2 internodes were used as material for introduction into in vitro culture.

During the first stage in the Laboratory of Biotechnology of the National Laboratory of Agroecology of the Russian Academy of Sciences, sterilization modes of C. aphyllum explants with the listed preparations were investigated.

During the pre-sterilization phase, in vitro, the agents for surface treatment used in the standard sterilization protocols for tree and shrub species [11 -14] were applied [11 - 14] (Table 1).

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Table 1. Sterilizing agents used for explants' preparation of *C. Aphyllum.*

Name of the sterilizing agent	Manufacturer	Concentration, % v/v.	Composition of the drug
«Lysoformin 3000»	«Hygiene Plus» LLC, Russia	1.0; 5.0	Glutaraldehyde (9.5 %); Glyoxal (7.5 %); Didecyldimethylammonium Chloride (9.6 %)
Hydrogen peroxide solution H_2O_2	Federal State Enterprise «Plant named after Ya. M. Sverdlov»	10.0	Hydrogen peroxide (37.0 %)
«Belizna - econom»	PK «Rusbytkhim» LLC, Russia	10.0	Sodium hypochlorite NaOCl (5.0 – 15.0 %)
Silver nitrate solution $AgNO_3$	Dia-m, Russia	0.1; 0.2	Silver nitrate, reagent grade, мin. 99.0%
«Fundazol»	Agro Kemi, Hungary	1.0	Benomil, 500.0 g/kg

For pre-sterilization treatment, explants were collected in gauze bags of 10 pieces and washed in a soapy solution for 10–20 minutes, followed by their washing in running water for an hour [15]. At the first stage of sterilization, 70% ethyl alcohol was used (treatment time: 60 seconds), after which the explants were treated in basic sterilizing solutions, the concentration and exposure time of which were selected experimentally. After the basic sterilization, the explants were washed five times in sterile distilled water, and one was put in vials with a nutrient medium according to the Murasige-Skoog protocol (MS). All manipulations were carried out in sterile laminar box conditions.

The cultivation was carried out on the STELLAR-PHYTO LINE (Russia) at a photoperiod of 16/8 h (day-night), an intensity of illumination 80 - 85 µmol m^{-2} s^{-1}, and temperature of a 22 - 24 ºC. Preparation and sterilization of the nutrient medium were carried out in accordance with standard protocols [16]. During the experiment, a percentage of sterile, unviable, and regenerating explants was recorded.

In the next step, drugs (Table 1) were used to test their action on five bacterial and one fungal strain: 2.1, 2.2, 2.3, 6.2, 6.3, 6.4, all isolated from *C. aphyllum*.

Bacterial suspensions of the tested strains in sterile saline solution of 0.89% NaCl were prepared for testing the cultivated regenerants for microorganism contamination. To control the sterility of the treated agents, samples were seeded onto plates of sterile nutrient agar (medium for determining the quantity of mesophilic aerobic and optional anaerobic microorganisms (KMAFAnM), production of VNIIMS, Uglich) in petri dishes. Cultivation was carried out in the thermostat TS-1/80 SPU at a temperature of 28–30 ºC. Solutions of sterilizing agents in the necessary concentrations were prepared in aseptic laminar box conditions using sterile distilled water. Liquid and dense potato-sucrose medium and liquid Chapek medium (produced by Hi Media Laboratories PVT, India) were used to separate microorganism crops.

Each of the samples was placed in a sterile test tube, 10 ml of sterile physiological solution was added, and periodically shaken for 10 – 15 min. After that, the resulting suspensions were taken from the tubes with a sterile pipette, 1 ml each, and added to the tubes with liquid potato-sucrose medium or Czapek's broth (9 ml) in three repetitions and left in a thermostat at a temperature of 28 – 30 ºC until 7 days to obtain accumulative microorganisms' cultures.

Pure strains were isolated by inoculation on Petri dishes with KMAFAnM medium and standard bacteriological methods were used [17].

To determine the inhibition zones, prepared sterile Petri dishes with KMAFAnM medium were infected with isolated pure cultures of microorganisms. To do this, one- or two-day bacterial suspensions with microorganisms' strains (depending on their individual growth rate on a dense medium) with a concentration of 5×10^{-8} CFU/ ml were added for 1 ml to Petri dishes with KMAFAnM medium to obtain in 24-48 hours microbial "lawn". After uniform distribution, the excess suspension was removed. Next, 4 wells (size 5 mm) were made in a Petri dish with a metal punch, where the same amount of the test preparation or solution was placed (3 drops of the drug). The result was recorded after 24–48 hours, depending on the growth rate of microorganisms [18]. The studies were carried out in 2-3 replicates.

The results were recorded by inhibition microorganisms zone size around the hole, similar to the size of the paper discs, including their diameter. If the inhibition zone is 15 - 25 mm, the microbes are sensitive to the substance, up to 15 mm are not sensitive; the absence of such a zone indicates the resistance of the bacterial culture to the substance (no sensitivity) [17].

To determine the drug effect on the fungal strain 6.4, a study was conducted in the liquid Chapek medium. Agar blocks with a diameter of 0.5 mm were used for infection (they were cut with a metal sterile puncher from a mushroom strain cultivated on a dense nutrient medium Chapek for 7 days). The drugs were prepared with a knowingly increased concentration of 10 times, then 0.5 ml in 4.5 ml of Chapek medium was added to the vials in three repetitions. There, in aseptic conditions, disks containing fungi culture were placed with tweezers and left in a thermostat at 30 ºC for 7-10 days to detect the positive or negative effect of the drug. The result was assessed visually by the presence or absence of fungal growth in the vial. The experiment was carried out in two times.

All results were statistically processed with the help of the Microsoft Excel program package and were presented in the arithmetic average error form. The growth delay zones' size was determined by the ImageJ program (CWA), the differences are valid for $p <0.05$.

3 Results and discussion

3.1 Surface sterilization of explants *C. aphyllum*

There were 11 sterilization modes of *C. aphyllum* explants applied and studied for aseptic cultivation, and the following results were obtained (Table 2).

Table 2. The influence of different sterilization's modes on regenerants *C. Aphyllum*.

Number of sterilizing modes	Sterilization drug and used concentration, % v/v	Exposition, min	Efficiency of sterilization, %
No.1	Ag NO3, 0.2	10	80.01
No.2	«Lysoformin 3000», 1.0	5	80.81; 40.02
No.3	«Lysoformin 3000», 1.0	7	10.02
No.4	«Lysoformin 3000», 1.0	10	20.02
No.5	«Lysoformin 3000», 1.0 + H2O2, 3.0	4 +2	30.02
No.6	«Belizna - econom», 10.0	10	18.22
No.7	H2O2, 10% + «Lysoformin 3000», 1.0	5 +5	30.02
No.8	«Belizna - econom», 10.0 + +«Lysoformin 3000», 1.0	5 + 5	45.42

No.9	H2O2, 6.0	10	0.02
No.10	H2O2, 6.0	15	80.02
No.11	H2O2, 10.0	5	81.82

[1] June 2022, when introduced into in vitro culture
[2] August 2022, when introduced into in vitro culture

The highest percentage of sterile explants were observed under modes No. 1 and 2 (tested in June), as well as modes No. 10 and 11 when introducing *C. aphyllum* in August in vitro. There is no further regeneration of explants in sterilization mode No. 1. 8.3% of regenerated explants were obtained in mode 2. Obtained 40% and 36.3% of regenerants in modes 10 and 11, respectively. Other modes show minimal or no regeneration. Reducing the surface sterilization time from 15 minutes to 5 minutes for mode No. 11 has the positive effect of saving time at the surface sterilization stage with almost a comparable percentage of regenerated experimental samples. The introduction's time into in vitro culture and the condition of the explants themselves also play an important role (Figure 1).

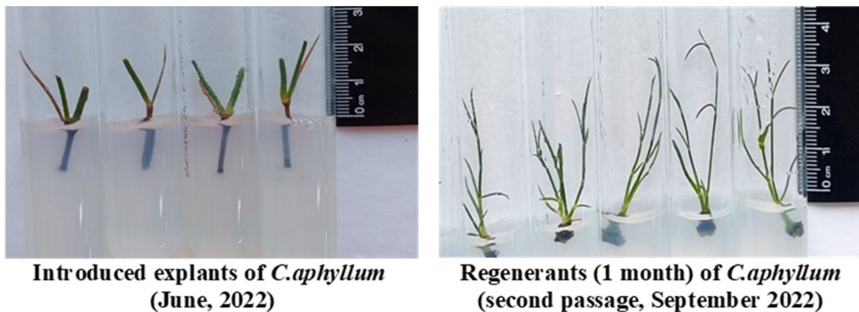

Fig. 1. Introduced explants and regenerants of *C.aphyllum.*

Comparing the percentage of sterile and non-sterile explants, it can be argued the introduction to the culture in vitro in August gave a smaller positive effect with the introduction in June, since *C. aphyllum* microcuttings were introduced partially lignified in August, the viability of such samples is usually lower [19].

3.2 Study of the sterilizing agents' effect on the isolated microflora from *C. aphyllum* explants

Three bacterial strains (2.1, 2.2, and 2.3) were isolated from the samples introduced in vitro in June after 7 weeks of cultivation in artificial conditions.

Researchers confirm that with further cultivation or transplantation, the number of regenerants affected by bacterial infection increases while the fungal population decreases; the «hidden» bacterial microflora can manifest at passages 4 and later [20, 21]. From samples introduced into the culture in vitro in August, after 10 days of cultivation, 4 strains of microorganisms were isolated: 3 bacterial (6.1, 6.2, and 6.3) and 1 fungal (6.4). This result confirms the conclusion of the researchers [20] that at the initial stage of introduction to the culture in vitro, there is infection with fungi. It can be explained that a small amount of persistent spores could remain after treatment in samples, for example, in the vascular system of plant organs during surface sterilization with the tested drugs [21]. In a study to determine the effect of the drugs used on the isolated fingi strain 6.4. determined the efficiency of such drugs as «Lyzoformin 3000» (1% and 5%), solutions H_2O_2 and «Belizna - econom» (10%),

to which the studied strain showed sensitivity. After cultivation for 10 days, there was no growth in seeding into the Chapek broth (Figure 2).

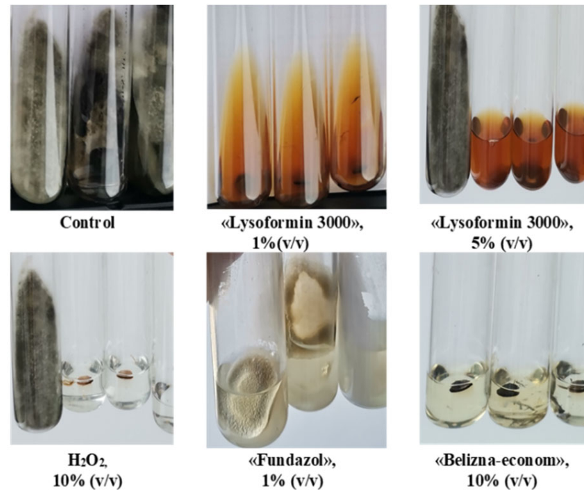

Fig. 2. The effect of various preparations on the fungal strain isolated from *C. aphyllum* explants.

In contrast to the above sterilizing agents, the drug "Fundazol", used as a fungicide for the treatment of fungal infections [22], had no effect on the fungal strain isolated from the *C. aphyllum* explant. The presence of microorganism growth in a liquid medium with the addition of this drug at a concentration of 1 % (Figure 2) was not affected.

Thus, the drugs «Lyzoformin 3000» (1% and 5%), hydrogen peroxide H_2O_2 (10%), «Belizna - econom» (10%), delayed the growth of the fungal strain 6.4. and can be recommended for sterilization treatment with longer exposure time.

Conducted studies on the action of sterilizing agents on bacterial strains isolated from *C. aphyllum*, showed differences in the sensitivity of microorganisms' cultures to the studied drugs. The results for growth delay zones of microorganisms by diffusion from holes to agar are presented below (Figure 3).

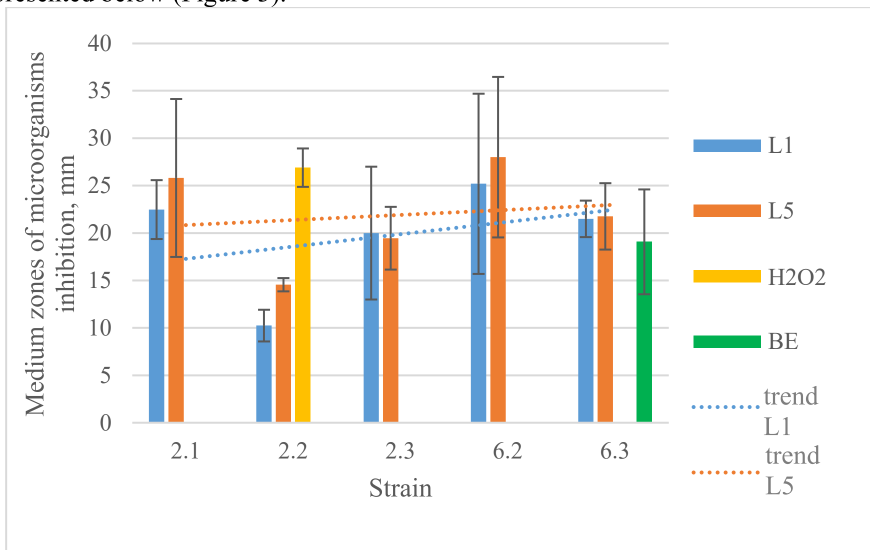

Fig. 3. Efficiency of sterilizing agents' action on isolated microorganisms.

The drug "Lysoformin 3000" at concentrations of 1.0 and 5.0% (v/v), acts on all isolated microorganisms. The interval of the drug's action on bacterial strains at a concentration of 1% ranged from 10 to 25.2 mm; at a concentration of 5%, it ranged from 14.9 to 25.8 mm, which confirms the average sensitivity of strains to the influence of the studied concentrations. As the trend lines for «Lysoformin 3000» show, a 5-fold increase in its concentration does not lead to a significant increase in the average zones of growth inhibition of the studied bacterial strains. An increase in the concentration of "Lysoformin 3000" up to 5% can be recommended if the treatment time is reduced, since the trend lines for the concentrations of 1% and 5% (Figure 1) are close, and the decrease in time when using a more concentrated solution of the drug is consistent with the data of Kritskaya T.A. and Kashin S.A. [11].

Only one strain of 2.2 was sensitive to hydrogen peroxide solution (10%). In the remaining cases, no delay zone solution was found, or the effect was short-lived, and after 24 hours secondary growth was found on the Petri dishes, thus the solution did not affect all bacterial cells of the strain under study.

The average sensitivity to the drug «Belizna-econom» (10%) was shown by the bacterial strain 6.3 (growth delay zone was 19.08 mm), and this drug did not affect the rest of the crops—growth delay zones were not detected.

All isolated strains were not sensitive to the 0.1% silver nitrate $AgNO_3$ solution. Thus, this solution does not act in the studied concentration on the microflora isolated from *C. aphyllum,* and the delay of growth of microorganisms on a dense nutrient medium has not been detected.

4 Conclusion

The results of research allowed to establish the possibility of top-level surface treatment of *C. aphyllum* explants for further regeneration in vitro for the first time. The optimum mode of sterilization of 10% - hydrogen peroxide solution for 5 minutes after preliminary exposure in 70% ethanol within 1 minute was selected for *C. aphyllum* microcuttings, and regeneration of the explants was 36.3 percent. Reduced H_2O_2 solution concentration to 6% and increased sterilization time by 3 to 15 minutes gave almost the same percentage (40%) of regenerated samples.

As a result of *C. aphyllum* explants' test for microbial infection, it is determined that the preparation «Lysoformin 3000» has an overwhelming effect on the isolated strains of microorganisms. 80% of bacterial strains were not sensitive to the 10% concentration of «Belizna-econom» and 10% H_2O_2 solutions, and a solution of silver nitrate $AgNO_3$, 0.1%, did not act on bacterial strains from *C. aphyllum* explants. The selected fungi strain 6.4 showed sensitivity to all the listed drugs, except «Fundazol» at a concentration of 1%. The partial results of the study, taking into account the influence of sterilizing agents on microflora, could be used to draw up a full protocol for the sterilization of *C. aphyllum* during the introduction stage of culture in vitro.

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