Development of Clonal Micropropagation Technology for Ludisia Discolor (Ker Gawl.) A. Rich. *In Vitro* Conditions

Vladimir Nazarov[1,*], *Ekaterina* Semenova[2], *Elena* Andronova[2], *Inna* Tsokalo[1] and *Elena* Semenova[1]

[1]V.I. Vernadsky Crimean Federal University, Simferopol, Russia
[2]Komarov Botanical Institute of the Russian Academy of Sciences, St. Petersburg, Russia

Abstract. The features of clonal micropropagation in three variations *of Ludisia discolor were studied.* Brood buds, apical shoot meristems, immature seeds and mature seeds were used as explants. They are grown using Murashige & Skoog modified medium. A special technique has been developed for the sterilization and planting of microscopic mature seeds. Mature seeds germinated for 60-70 days. Seeds from 25-day-old capsules showed the best results among immature seeds. They gave mass shoots already on the 25-30 day after planting. The roller of the future first leaf of the protocorms was laid in the dark 2 months after germination. Switching to the light mode induced rapid gemmorizogenesis - the formation of the first green leaves, stem and adventitious roots. Seeds from 15-day-old capsules did not germinate at all. Planting with immature seeds is the most effective method of clonal reproduction of *L. discolor in vitro.*

1 Introduction

It is known that the tissues of natural and cultivated plants of terrestrial orchids from the genera *Anoectochilus* and *Ludisia* contain a high content of various glycosides, polysaccharides and flavonoids, which have significant bioactivity (Poobathy et al., 2019, Yan-bin Wu et al., 2020). In China, almost all species of *Anoectochilus* and Ludisia are used as folk medicine. Among these species, *A. roxburghii* (Jinxianlian in Chinese) is considered the most famous and popular medicinal and edible species of *Anoectochilus* in China. Fresh or dried whole plant *A. roxburghii* is mainly used for the treatment of diabetes, hepatitis, hypertension, tuberculous hemoptysis, fever, rheumatism and rheumatoid arthritis, pleurodinia in China (Huang, 2006, Huang et al., 2007, Ye et al., 2017, Zeng et al., 2017). Species from the genus *Ludisia* are also valuable raw materials for the production of flavonoids, anthocyanins and antioxidants (Poobathy et al., 2019).

Anoectochilus roxburghii is officially designated as the only source of Jinxianlian in the "Standards of Chinese Medicinal Materials of Fujian Province in China" (2006 edition) (Huang, 2006). Popularly, other Anoectochilus species are also called Jinxianlian in local herb markets due to similar traditional medicinal efficacy, including antipyretic effect and

* Corresponding author: vvn22222@mail.ru

detoxification, expelling wind and eliminating dampness (Ye et al., 2017). In addition, some clinical applications of Ludisia species are similar to those of *A. roxburghii* in the treatment of tuberculosis (tuberculosis hemoptysis), rheumatism, rheumatoid arthritis, snake bite, etc. (Ai, 2013).

The demand for *A. roxburghii* raw materials in the pharmaceutical market is growing every year due to its unique medicinal and edible properties. Such extensive consumption has led to a sharp reduction in the natural reserves of *A. roxburghii* to a critical level. Therefore, active research is currently underway to find suitable substitutes for officially registered species for the production of Jinxianlian based on *Anoectochilus* or other related genera. (Yan-bin Wu et al., 2020). However, the introduction of new *Anoectochilu*s and *Ludisia* species into circulation will not fundamentally solve the problem of the shortage of the resource base of Jinxianlian medicinal raw materials, since intensive collection of raw materials from natural populations will quickly deplete this new resource base and put these species on the verge of extinction. *Lidisia discolor* is also intensively harvested at home as a valuable medicinal raw material for traditional medicine (Ranjetta et al., 2018). In addition, *L. discolor* is actively withdrawn from natural habitats by plant collectors as "precious orchids", which are very popular among orchid collectors because of the unique features of coloring, texture and unique mosaic pattern of leaves. Currently, *L. discolor* is the leader in the number of specimens in private collections among other "precious orchids".

The only effective solution for expanding the resource base and preserving natural reserves is the development and introduction into production of biotechnologies for growing these valuable orchids. However, in this way of solving this problem, it is necessary to study a number of issues. The first of them is connected with the complexity of seed reproduction of these rare orchids. Specialized pollinators are needed for seed production, since the morphology of the flowers is unique. The seeds of these orchids are completely full of spare nutrients and germinate only with the help of specialized mycorrhizal fungi. Moreover, the seedlings of these orchids have been living exclusively underground for several years and feed on mycorrhizal fungi. These features of reproductive biology must be carefully taken into account when developing biotechnological methods of growing medicinal plants outside of natural populations, i.e. where there are no natural consorts - specific insect pollinators and mycorrhizal fungi.

Cultivation of *Anoectochilus* and *Ludisia* species in interaction with specific mycorrhizal fungi is of fundamental importance for obtaining high-quality medicinal raw materials from these orchids. The few studies of the features of early development in some species clearly indicate that the level of accumulation of glycosides, polysaccharides and flavonoids in plants grown under sterile conditions in vitro and in the presence of mycorrhizal fungi is very different. It is likely that the accumulation of biologically active compounds in the tissues of orchids is a kind of immune response to the penetration of mycorrhizal fungi into the tissues. Therefore, two-stage biotechnology is of interest for the production of high-quality medicinal raw materials on an industrial scale.

Methods of mass reproduction of this rare plant under in vitro conditions can act as one of the elements of such technology. Therefore, the purpose of this study was to study the features of the course of clonal micropropagation of *Ludisia discolor*. To achieve this goal , it was necessary to solve the following tasks: 1) to study the behavior of various explants on the nutrient medium; 2) to develop an effective method of sterilization and seed planting.

2 Materials and Methods

Three variations *of Ludisia discolour* from the natural habitats of Southeast Asia were used as the object of research: var. *nigrescens*, var. *dawsoniana*, and var. *dawsoniana* f. *variegate*, which differed among themselves in color, mosaic and texture of the leaves (Fig. 1). The collection material was obtained from the tropical orangery of the Botanical Garden of the Botanical Institute named after V. L. Komarov RAS, St. Petersburg. Subsequently, these plants were cultivated for two years in the tropical greenhouse of the Crimean Federal State University named after V.I. Vernadsky, Simferopol. Brood buds, apical shoot meristems and seeds were used as explants.

A modified Murashige & Skoog medium was used for planting (Murashige & Skoog, 1962). Unlike the standard version, it had half the amount of mineral components, sucrose at a concentration of 15 g/l. Phytohormones 6-BAP and indoleacetic acid were used in the ratios 1:2, 1:1, 2:1.

Fig. 1. Variability of the studied forms of Ludisia bicolor in color, texture and mosaic pattern of leaves: 1 – *Ludisia discolor* var. *nigrescens*, 2 – *Ludisia discolor* var. *dawsoniana*, 3 –*Ludisia discolor* var. *dawsoniana* f. *variegata*. *Source:* Compiled by the authors.

To obtain seeds, we carried out geitenogamous pollination between flowers of the same inflorescence. The full ripening of fruits in all studied variations of *Ludisia discolor* in the greenhouse conditions occurred on 30-35 days.

Mature seeds of *L. discolor* have microscopic dimensions. Therefore, a specially developed technology of sterilization and planting was used for their inoculation of medium *in vitro*. It was based on a method that was developed by E.V. Andronova et al. (2007) for sterilization of mature microscopic orchid seeds with a chemical solution using a syringe and a special fine metal mesh trapping (personal message). We did not use a metal mesh to trap the seeds. *L. discolor* seeds were placed in filter bags (paper bags from a coffee filter). A portion of dry mature seeds was poured into such a bag and they were covered with a round paper circle with a number. The diameter of the circle was slightly smaller than the diameter of the piston of the syringe. The tight fit and the presence of a paper circle according to the size of the piston contributed to a good mixing of the contents of the bag with the sterilizing liquid during the movement of the piston. The finished bag was tied and placed in a syringe.

A mixture of distilled water and a 10% solution of sodium hypochlorite in a ratio of 1:1 was used as a sterilizer. Sterilization lasted 20 minutes. The procedure of washing the seeds from the sterilizer was carried out in the same way in distilled water 2 times for 10 minutes. With sterile scissors, the tail part was cut off from the bag. The head part of the bag was completely unfolded and transferred to a Petri dish with a nutrient medium.

Planting *Ludisia discolor* with mature seeds from already opened capsules turned out to be a technically time-consuming operation. Therefore, we tested the technology of planting this orchid on a sterile nutrient medium using immature seeds from unopened capsules according to the method of P. J. Kauth et al. (2008).

3 Results and discussion

Affiliations In the first stage from *Ludisia discolor* var. *dawsoniana* capsules of different ages were taken to determine the optimal stage of seed development for planting. In these studies, 15, 20 and 25-day capsules were used from the moment of initial pollination. All the capsules were planted on the medium from one flask. Petri dishes with planted seeds were kept for the first 60 days in a dark thermostat at 18-20 °C. Seeds from 25-day capsules showed the best germination (Fig. 2.1). Initially, the seed embryos swelled and acquired a milky white color. A month after planting, the protocorms massively tore the seed coat from the seeds and the first root hairs formed in their basal part (Fig. 2.2). After 2 months, the protocorms formed an apical roller of the future first leaf. The basal part by this time was already covered with several rows of root hairs (Fig. 2.3).

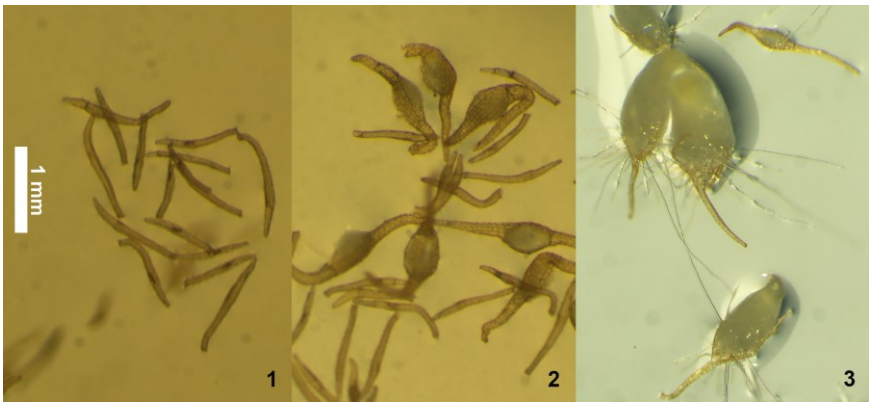

Fig. 2. Seed germination and haemorrhizogenesis of protocorms in *Ludisia discolor* var. *dawsoniana* in the dark phase: 1 – immature 25-day seeds after planting on a nutrient medium; 2 – mass germination of seeds and the release of protocorms from the test, 40 days; 3 - the basal part of the protocorms is covered with several rows of root hairs, a roller of the future first leaf is laid in the apical part, 60 days. The number of days is indicated from the moment the seeds are planted on the nutrient medium. *Source:* Compiled by the authors.

After 60 days from the moment of planting, the protocorms were transferred to a growth cabinet with an 8-hour lighting period and a temperature of 25 °C. After 5 days, there was a massive greening of the protocorms (Fig. 3.1). After another 5 days, the leaf blade of the first leaf began to form from the apical roller (Fig. 3.2). After a month of keeping crops in the light, the seedlings had the development of 2-3 stem metamers with root hairs (Fig. 3.3).

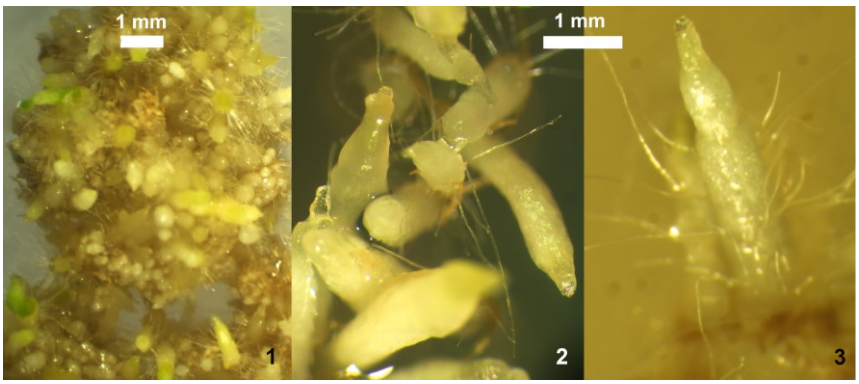

Fig. 3. Gemmorizogenesis in seedlings of *Ludisia discolor* var. *dawsoniana* in a growth cabinet with an 8-hour lighting period: 1 – greening of protocorms and seedlings, 65 days; 2 – the beginning of leaf blade formation, 70 days; 3 - metamerization of shoot in seedlings, 90 days. The number of days

is indicated from the moment the seeds are planted on the nutrient medium. *Source:* Compiled by the authors.

Seeds from 20-day capsules germinated much worse. In a dark thermostat on the 30th day after planting, only about 20% of the sown seeds swelled or formed protocorms. In size, the protocorms were smaller than the protocorms from 25-day-old seeds. The relative delay in the development of seedlings persisted even when kept in a growth cabinet. Seeds from 15-day-old capsules showed no signs of germination during the entire observation period in a dark thermostat and growth cabinet.

In the other studied variations of *Ludisia*, seeds were taken for planting only from 25-day-old capsules. Seed germination in the dark phase and gemmorizogenesis in the growth cabinet occurred in them similarly to the first variation.

Previously obtained data have been confirmed that the induction of brood bud development in *L. discolor* under *in vitro* conditions is not always successful and depends on the optimal concentration of phytohormones in the nutrient medium (Ying et al., 2021). In addition, the introduction of brood buds into culture *in vitro* is often accompanied by the development of fungal and bacterial microflora, despite the harsh treatment of explants with various sterilizers (Poobathy et al., 2019). The application of the developed methods of sowing mature and immature seeds for clonal micropropagation allows us to increase the multiplication coefficient of *L. discolor* by thousands of times.

It was previously shown that in order to preserve rare species of polymorphic orchids, an integrated approach based on population monitoring, research of intraspecific structure, as well as features of biology of both seed and vegetative reproduction should be applied (Shirokov et al., 2020). Our studies of another rare polymorphic orchid, *Ludisia discolor*, have shown that when seed propagation is carried out *in vitro*, combined methods of planting with various types of explants should be used: brood buds, immature seeds and seeds from opened capsules. At the same time, the choice of a specific type of explant depends on the general tasks - maintaining the genetic diversity of the population or multiplying a rare variation. Whereas when choosing the method of planting seeds, it is necessary to take into account the possibility of repeated exits in the population for collecting material and the timing of its delivery to the laboratory.

4 Conclusion

The features of the course of clonal micro-propagation of Ludisia discolor using various types of explants: brood buds, mature and immature seeds in vitro on a standard Murashige & Skoog medium have been studied. It has been established that the use of immature seeds from 25-day-old capsules is the most effective for mass reproduction of this rare orchid. The use of mature seeds from already opened capsules is limited by the difficulties in their sterilization due to their microscopic size. We have developed an effective method of sterilization of small orchid seeds. It also facilitates the subsequent planting of such seeds on nutrient media. However, its success depends on the accuracy of the choice of the stage in the development of seeds, which can only be done in greenhouses. When taking material from natural populations, mass planting using mature seeds is more reliable, but sterilization and planting of such seeds is much more difficult. A special technique is required, which greatly simplifies sterilization and planting. Thus, for the first time in the Russian Federation, a fundamentally new technology of clonal micropropagation of Ludisia discolor has been developed in vitro.

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