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Use of biotechnological methods for the Albanian genetic resources

E. Kongjika, Zh. Zeka, E. Çaushi, I. Stamo, F. Babani[1]

Summary

The authors present the potential opportunities of the "*in vitro*" culture for the use of the Albanian genetic resources of the spontaneous and cultivated plants. The study estimates several techniques that have a good impact on agriculture, forestry, fruit-cropping, floriculture, etc. In the context of serious transitory conditions in Albania, that are reflected in the massive deforestations, loss of fruit orchards, decrease in agricultural production etc., the authors support possible strategies for the use of "*in vitro*" culture techniques by way of experimental studies on: micro propagation of embryo culture for the forest species threatened to disappear (walnut, Valona oak and holm oak) as a useful alternative for the rapid reforestation; micro propagation and genetic improvement of rare plants in the Balkan region (i.e. *Ramonda serbica* Panc.) so as to cultivate them as decorative plants of rocky gardens; the recovery of carnation, its autochthonous varieties, and potato by way of meristem culture for the production of virus-free plants. Plants were tested by means of indicators and induced fluorescence; the regenerating ability of wheat anthers among 20 Albanian cultivars in order to obtain haploid culture of wheat anthers. The paper reports the results obtained and outlines future fields of study.

Key words: Micropropagation, meristem culture, embryo and anther culture.

1. Introduction

The *in vitro* culture techniques of plant tissues have a great interest for the research and practical work in the area of plant physiology, plant breeding, germplasm conservation, etc. The advantages of using those methods are:

fast, effective and ever-seasonal propagation of elite clones, selected for practical interests, as well as autochthonous plant species;

production of clones free from diseases, especially viruses;

the genetic improvement of the cultivated plants, also using wild species and genetic resources in the gene introgression;

the establishment of *in vitro* collections for germplasm preservation of wild, endemic, rare and threatened plants.

[1]Institute of Biological Research, Academy of Sciences, Tirana (Albania).

The study highlights the contribution of various *"in vitro"* techniques in solving out the current problems related to safeguard of genetic resources.

2. *In vitro* techniques applied to forest species

Walnut, Valona oak and holm oak have been studied as autochthonous forest species threatened to disappear.

Walnut (*Juglans regia* L.) embryoculture

Walnut is considered as an autochthonous and cultivated tree throughout Albania up to the height of 1200 m. It originated from South-Western Asia, spontaneously spread especially in the valley of Shkumbin, from Librazhd to Prrenjas, in Martanesh, Gramsh, in the North-East of Albania and in Korça. Walnut is widely used as a very delicious fruit, rich in oil, proteins, vitamins, etc; as a very precious wood for furniture; its leaves and bark are used as medicinal and dyeing essence. It has been severely damaged over the recent years and it is included in the list of the endangered plants. (Vangjeli *et al.,* 1995).

The techniques of sexual propagation for walnut produce plants of large variability, which makes the production of uniform clones impossible. In addition, the asexual and conventional propagation is not useful to pruduce original clones of industrial quantities.

The interest for walnut *in vitro* culture has increased late due to the rooting difficulties. Micropropagation can be used in the future for the genetic improvement of walnut and the production of new clones of agricultural interest. Propagation of parent stock by embryo cultures is evaluated with priority for the rapid cloning of the varieties bearing specific values and walnut elite plants. In our study, we started to inoculate explants following, with little adjustments, the methods by Rodriguez (1982) and Cossio and Minotta (1985). The walnut fruits swelled in 24 and 48 hours and were disinfected with 0.1% $HgCl_2$. The walnut zygotic embryos, without or with some cotyledons, were inoculated in a MS medium enriched with 2 mg l^{-1} glycin, 0.5 mg/l of nicotynic acid, 50 mg/l of pyridoxine HCl, and 100 mg/l of mio-inositol. Four treatments were used considering different concentrations and types of phyto-hormones:

a) *without phyto-hormones*; b) *Benzilaminopurine* (BAP 1mg/l); c) *Kinetin* (4 mg/l) and d) *Zeatine* (2 mg/l). Sucrose was used in quantities of 20 g l^{-1}, 0.6% agar, whereas pH was 5.5. With regard to sub-cultures, the basic nutritional MS medium was added with BAP 0.09 mg l^{-1}P, while for rhyzo-genesis the quantity of mio-inositol increased to 200mg l^{-1}. The cultures were incubated for 16 hours and lighted by white fluorescent lamps with 2000 lux, at the temperature of 25 ± 2^0C.

The data showed that the 24 hour-swollen explants bear the highest sprouting index as compared to the 48 hours ones. The comparison of the germination rate among different treatments showed that this indicator was very high due to the presence of zeatine cytokine. The germination rate was 0 for the treatment without phyto-hormones. The presence or absence of cotyledons, was an essential prerequisite for the differences in the germination rate. Only 23% of embryos without cotyledons were inclined to further development, whereas the highest percentage of germination was found in the zygotic embryos with the cotyledons.

The research indicated some phases of morphogenesis for walnut embryo. The formation of embryo-genetic tissue leaded to the appearance of callus with hard vacuolated cells, having different forms and sizes in the bottom and central part and homogenous and compact cells in the other parts. In different parts of callus, in particular among parenchymatic cells, there were embryo-genetic knots that formed new buds during the process of organogenesis.

After four weeks the buds and the callus were transferred into a new medium, while after 6-7 weeks the first sub-culture ended. As a result of micropropagation over 8 weeks, the coefficient of propagation was 3 and after three months it was 6. The transfer to the rhyzogenesis sub-cultures stimulated the formation of new rooted plants, ready for acclimatisation. The embryo stages of walnut culture accompanied by micropropagation are shown cyclically in Fig. 1.

Micropropagation of Valonia oak (*Quercus macrolepis* Kotchy) and holm oak (*Quercus ilex* L.)

Valonia oak forms forests 800-900 m above the sea level in Albania, mainly in the South (between Konispol and Vlora Bay) as well as around some villages in the zones of Shpirag and Molisht. The simple forests of Valonia, oak are sparse and planted with dense evergreen shrubs. Man uses Valonia oak for tannin. Valonia oak is getting sparse and in some places it's prone to disappear as a result of its uncontrolled use and because of intensive agricultural activity in correspondence of hilly area. The intervention in degraded forests by way of reforestation will considerably improve the structure of that very important vegetation of the south coast of Albania (Vangjeli, 1993).

The Holm oak is precious both as wood for agricultural and marine tools and as a decorative plant. It grows in the Mediterranean forests and shrubs in the *southern* and *central* part of Albania, at the height of 1,000 m. It is considered to be Steno-Mediterranean. Today, it's very rare, due to human activity and frequent fires (Vangjeli *et al.*, 1995).

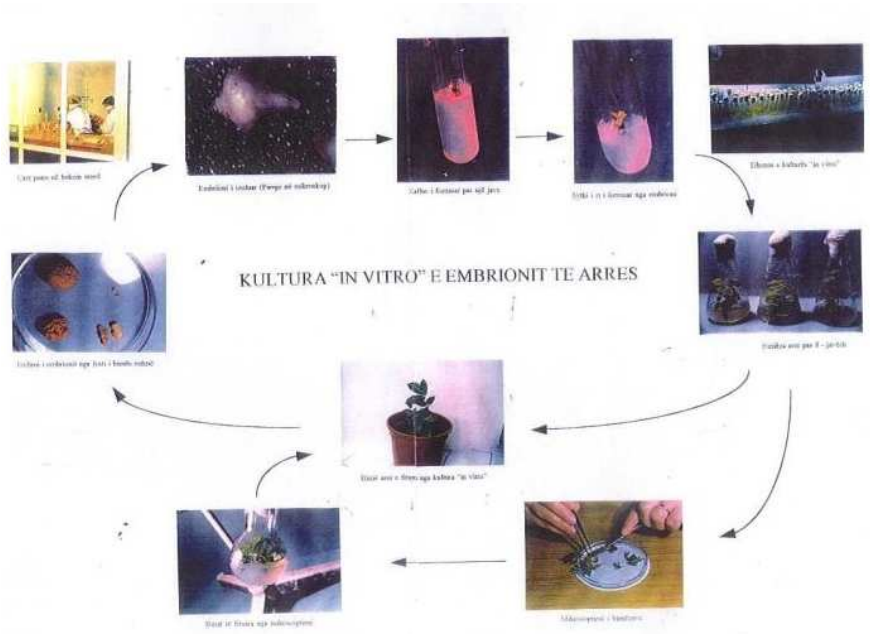

Fig. 1. *In vitro* culture of walnut embryo

The vegetative propagation of the selected lines is an effective method for the production of clones and the breeding of those trees. Though some scientists have faced difficulties in *in vitro* culture because of tannin production, others have managed to successfully use the propagation by explants or somatic embryogenesis by embryo axis (Shoyama *et al.*,1992). Below are shown some preliminary data on the micropropagation of explants of *Valonia* and *holm* oaks. One year or 6 month old *Valonia* and holm oak plants were used, produced by seeds germinated in screenhouse. Knots 20 mm long, with lateral buds and meristems were cut. Disinfection was made only for walnut embryo. MS medium was diluted twice by adding (in $mg.l^{-1}$) 100 mio-inositol; 0.5 nicotinic acid; 0.5 pyridoxine HCl; 0.1 thiamine HCl; 2 glycine; 30 g l^{-1} sugar, 0.6% agar. Two different concentrations of phyto-hormones were used: a) 2,4-D 0.1 mg l^{-1} and b) 0.1 mg l^{-1} of 2,4-D (acid 2,4- dichlorophenoxyacetic); combined with 5 mg l^{-1} of BAP and 1 mg l^{-1} of IAA (indol-acetic acid).

In all the above cases the explants produced calluses of different forms and size with colour from beige to green. By comparing the elementary growth of the explants it was observed that one-year-old plants reacted better than the juvenile ones. Among the meristems, that appeared to be more developed than the knots, it was observed that they primarily produced calluses, then appeared the embryos and finally the new buds. During the process of organogenesis, developed green plants with lateral twigs were formed (Fig. 2).

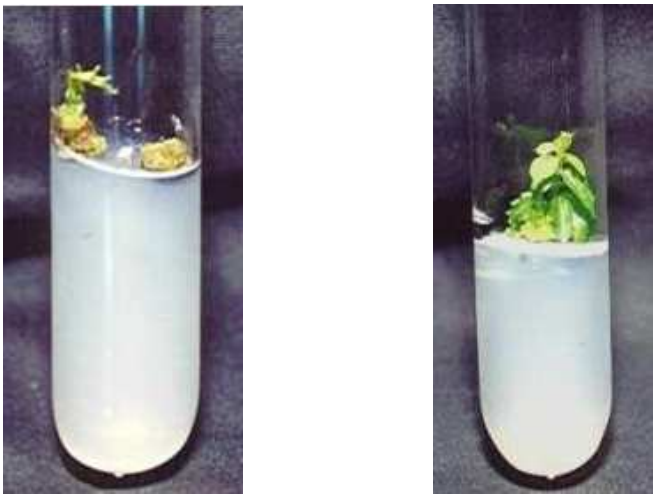

Fig. 2. Examples of *in vitro* culture on Valona and Holm oak

While comparing the plants grown with different phyto-hormones the development was stimulated when two auxins (2,4-D and IAA) and cytokinin BAP were combined. Such a combination promoted not only the formation of callus, but also the fast formation of new embryos and buds. The comparison of index for the new plants formed out of *Valonia* and *holm* oak explants indicated that the former reacted more effectively towards the "*in vitro*" culture. Embryos for the two forest trees were inoculated in the following experiments, which according to the literature (Shoyama *et al.*, 1992), were considered as the most effective elementary explants for the production of *Valonia* and *holm* oak plants.

3. Micropropagation of wild, rare and endemic plants

Ramonda serbica Panc., a Balkan plant and a European relic, belonging to the tertiary of Gesneriaceae family, was studied. According to Vangjeli *et al.*, 1995, this species is considered to be a rare plant in Albania, and therefore it should be preserved by way of cultivation as a decorative plant. It is characterized by rosette leaves, with 1 to 3 beautiful violet and lilac flowers of yellow in the *midst* and blue in the anthers. It also is characterized by resistance towards extreme drought and frost. It grows in strips of land, in the splits of calcareous rocks (Fig.3) and on the North or North-Eastern slopes, at 300-2000m above sea level. Due to its decorative style, it is preferred as a decorative plant in the alpine gardens of North European countries (Ferns, 1979). Plants suit properly well to the improvised rocks of the Botanical Garden of Tirana. Maps have been designed for the extension of the Balkan Gesneriades with *Ramonda serbica* Panc., typical for Albania (Meyer, 1970; Mullaj and Kongjika, 2000).

Fig. 3. Plants and example of formation of juvenile plants of *Ramonda serbica* Panc. by means of *in vitro* culture.

For this plant, we should apply a strategy of micropropagation and germplasm preservation based on "*in vitro*" collection.

As primary source of variation, seeds were collected from plants taken from six places in Central and North-Eastern Albania. Disinfection was made only with 70% ethanol. For all the stages we used the basic preparation of nitrate JG-B (Jungnickel, 1988) and pH 4.4-4.6. For the seed germination two treatments were set up: a) JG-B media without phyto-hormones and b) JG-B media by adding 1 mg l^{-1} of and 3 mg l^{-1} of ANA (-naphtalenacetic acid). During the second phase of new plant development, after 6 weeks, 4 treatment with phito-hormones were built (mg l^{-1}): a) JG-B media without phyto-hormones; b) 1 mg l^{-1} of JG-B and BAP; c) JG-B and 1 mg l^{-1} of BAP and 3 mg l^{-1} of NAA; d) JG-B and 0.5 mg l^{-1} of BAP and IAA. For the stage of micro-division, four treatments of the previous stages were used. Seed germination was reached at the temperature of 25^0C under continuous lighting. During the other stages, 18/24-hour photoperiod was applied.

During the first germination stage, it was observed that germination do not started simultaneously for the two treatments. When phyto-hormones were absent, germination starteds on the 12^{th} day, whereas in presence of cytokinin and auxin there was no sign of germination. The latter case showed that even when seedlings germinate, they were abnormal, chlorotic, just turning brown. This phenomenon might be explained by the oligotrophic characteristics of the seeds of this species, whose needs were satisfied by the basic JG-B medium, which was not rich in inorganic salts as compared to the universal MS. The positive influence of continuous light over the period of seed germination showed the positive photo-blastic properties of the seeds of *Ramonda serbica*. The time difference in seed

germination, according to the plant cultivation areas, was closely related to the seed growing conditions and maturity stage. These differences occured even in the late stages.

Only the juvenile plants of the first treatment, were subject to the second stage. The presence of several phyto-hormones led to the growth of juvenile plants. The plants without phyto-hormones passed to the normal organogenesis until reaching their typical physiognomy of separate plants of *Ramonda* with rosette leaves. Cytokinin BAP, present in treatment b and d (combined with auxin IAA), seemed to affect the formation and growth of lateral buds that led to the formation of grouped plants. The presence of low concentrated IAA affected rooting, while a high quantity of NAA damaged the juvenile plants.

The transfer of juvenile plants to the stage of micro-division allowed to have a high number of juvenile plants within four months. It showed that not only parts of organized plants, but also separate leaves might form complete plants, especially in treatment d where the quantities of cytokinin and auxin were equal. By way of micro-division, 10 juvenile plants generated out of only one plant.

These results have an importance for the preservation and use of *Ramonda serbica* Panc. and the establishment of "*in vitro*" genetic banks for wild, endemic and threatened plants. The final objective of this work will be to increase the ornamental values of this species by means of biotechnological methods, as well as its cultivation for market purposes as a plant of rocky gardens.

3.1. Meristem culture for plant recovering

Meristem culture, accompanied or not by the thermo-therapy of explants, is used with priority for the production of virus-free plants. 600 plant viruses are known, 80 of which are transmitted through sexual propagation; consequently the recovery of vegetable material should be improved. The method is based on the culture of meristem due to its active cell multiplication and absence of plasmodesmata which is thought to be the means of cell-to-cell movement of viruses.

Meristem culture of *Dianthus caryophyllus* L.

The above method was used for the propagation of carnation plants (*Dianthus caryophyllus*), which are widely spread decorative flowers in Albania with growing interest in the market economy. The autochthonous varieties were very beautiful, aromatic and colourful; whereas nowadays they suffer from frequent viruses.

When, we have isolated three kinds of meristem at the stereo-microscope: *a) without leaf primordia*; *b) with leaves and c) with two leaves*. Their diameter was 0.1 and they were 0.2-0.4 mm long. We used lateral and basal twigs, with juvenile characteristics over the period March-July. They were disinfected with 70% ethanol and 4% $Ca(ClO_3)_2$. The basic MS medium was used in several steps:

meristem culture with MS/2, adding 1 mg l^{-1} of thiamine, 0.1 mg l^{-1} of GA_3 (gibberellic acid), 0.1 mg l^{-1} of NAA, 20 g l^{-1} of sucrose and 0.6% agar;

microdivision in MS/2 without phytohormones. The preliminary sanitary assessment of plants was made by indexing onto herbaceous plants (*Chenopodium quinoa*, *C. amaranticolor*), by mechanical inoculation of the carnation plant material, extracted by buffer 0.03M Na_2HPO_4 in the presence of mercaptoethanol. Information was collected on the basis of the visual observation of symptoms such as yellow to brown lesions, necroses and leafroll.

During the first step, it was observed a vigorous development of meristems after 6 days and after 4 weeks the plants move to rhyzogenesis. Over the years, the success of meristems was 75-84%. Early spring, when the explants start their differentiating activity, is the optimore time for the explants. The selection of the 1:1 ratio (NAA : GA_3) seems appropriate for rapid obtention of juvenile plants during organogenesis of the explant. This is due to the effect of auxin in cell division of root primordia as well as with the effect of gibberellins in the growth of cells. Out of three kinds of meristems, only those having 1-2 leaf primordia grew satisfactorily, whereas those having no leaves didn't develop at all. It's obvious that the presence of leaf primordia gives good chances for organogenesis, but the minimum size of meristem makes the production of healthy plants certain.

Micropropagation is significant for the production of a considerable number of plants within a very short time. After four weeks, parts of the stem with double leaves produce rooted plants, which then pass into successive cultures. The presence of phyto-hormones is not necessary during this step, which decreases the cost of producing uninfected plants. The preliminary tests of phytosanitary control proved no symptoms of virus presence. The different steps of carnation meristem culture as a cycle are shown in Fig. 4.

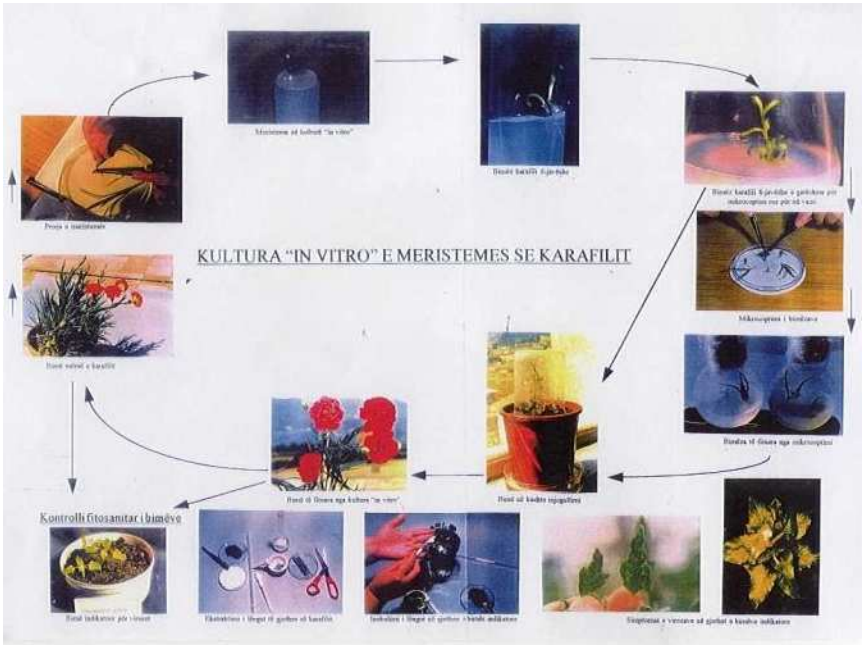

Fig. 4. *In vitro* culture of carnation meristems

Potato meristem culture

The use of this method for potato cultivars is under study, but was introduced in Albania long before. The classical system for the production of seed potato in Albania is slow (10 years), expensive and very dangerous for ecological and virus degradation of seeds. We have used the first two steps of the scheme to preserve germplasm at the International Potato Centre (CIP) in Lima-Peru (Espinoza *et al.*, 1984).

Tubers were taken as initial plant material, subject to preliminary processing, in order to break dormant buds. After disinfestations, the tubers were treated in two ways: a) Storage at a temperature of 25^0 C under normal lighting, and b) Preservation at 25^0 C for 20 days with photoperiods of 18/24 hours and 2 weeks prior to inoculation; the tubers were treated with 2 mg l^{-1} GA_3 per 10 min. The juvenile twigs were disinfected with 70% ethanol and 2.5% $Ca(ClO_3)_2$. Meristems were established in the MS medium and adding: 0.4 mg l^{-1} of thiamine HCl, 100 mg l^{-1} of mio-inositol, 2 mg l^{-1} of calcium panthoteins, 0.25 g l^{-1} of GA_3, 30 g l^{-1} sucrose and 0.6% agar. Calcium panthotein was used in two variants: a. dissolved in water and b. dissolved in 25% ethanol. The juvenile plants were transferred into the MS medium, which serves for rooting as well. This medium differs from the previous one for the increased amount of agar (0.8%). The newly formed plants were acclimatised in high atmospheric humidity, in a soil and perlite mix for two weeks and afterwards in Biza.

The juvenile plants and the tubers formed by "in vitro" culture were subject to phytosanitary control by using the fluorescence method (Lichtenthaler *et al.*, 1996). Xenon lamp with impulses serves as a means to induce fluorescence. Some parts of the fluorescence spectrum get excited by UV-A filter in front of the Xenon lamp (=340 nm). Images were recorder by video cameras that were synchronised in the system. The components operate under a PC programme.

The comparison of development for meristems out of treated tubers indicates that, based on the first treatment, only 10% of meristems were developed, whereas the presence of GA_3 developed 55% of them. In the water-dissolved calcium panthotein medium not a single meristem developed, which indirectly explains the vitamin decomposition in autoclaves. In cases when it was dissolved in 25% ethanol the percentage of the developed meristems was 45. The presence of GA_3 in the MS medium stimulated the increased number of meristems and the formation of juvenile plants in 4-6 weeks, whereas the combination of meristem culture and micro-propagation brought about a large number of plants (within 3 months, approximately 45 developed and rooted plants were produced out of one meristem). The period for each sub-culture was three weeks. When plants were kept in test-tubes for a long time (nearly 2 months), it's interesting to notice that they formed tubers that were subject to micropropagation. The preliminary data of phytosanitary control of leaves by way of induced fluorescence, indicated that its images on the 4 bands, as well as the data about fluorescence don't changed amongst samples. The variations of emitted values showed that the analysed plants were virus-free since florescence on the blue parts were much lower than on the red ones. Even for tubers there were no differences on the blue and green bands due to the presence of viruses.

Meristem culture for propagation is basic for the production of healthy planting material of potato in large amounts and in a short time, as well as for the future establishment of "in vitro" genetic bank for the preservation of potato genetic resources in Albania.

3.3. Haploid culture of wheat anthers

The production of double haploids is considered a potentially effective method for the set-up of homozygous lines, which after being crossed make way for fast stability of required qualities. Some initial experiments have been made by using 20 wheat genotypes (16 landraces and 4 from foreign countries) grown in greenhouses. Spikes were collected when microspores were in the single nucleus phase, i.e. from January to March,

and were stored in the dark at the temperature of 4-5^0 C for 4-10 days. Anthers were established in P_2 Potato extract (Chuang et al., 1978; Henry de Bayser, 1981). Calluses generated from the microspores (diameter 2-3 mm) detached from the anthers and were transferred into a differentiation medium.

The primary tests of some media (P_2, P_2G, Gl etc.) showed that the P_2 medium produces higher callus frequencies. More than 50% of wheat cultivars showed to respond to "*in vitro*" culture, but the cultivars Ciano Jubileo, Kamza 21, lines Agimi x C57 (190), Kamza 9 x Manital, responded much better to callus; Verry was used as a positive control. We should emphasize that the same genotypes showed a normal plant behaviour and resistance to diseases. Tests for the other stages of anthers' culture are in progress.

4. Conclusions

In Albania, given the difficult transitory conditions, there are problems of genetic erosion for wild and cultivated plants. The Authors of the present paper argue that the use of "*in vitro*" culture techniques by means of experimental studies might be an alternative strategy to tackle the above problems. These methods create conditions not only for the preservation of the existing biodiversity, but also for the use of plant genetic resources of the country and their use to human benefit.

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