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Via S. Camillo de Lellis, 01100 Viterbo

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"*Ex situ* conservation strategies of walnut and poplar genotypes by means of *in vitro* slow growth and cryopreservation methods"

Il relatore:

Prof.ssa Elena Kuzminsky

Il candidato:

Mohib Abdullah

Il correlatore:

Dr.ssa Emilia Caboni

Il controrelatore:

Prof. Maurizio Sabatti

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Summary

The present study was carried with two woody plant species i.e. *Juglans regia (*cv. Sorrento) and *Populus alba* (clone 6k3*)*. The main objective of the study was to develop an *in vitro* preservation protocol for *P. alba* and *J. regia*. The research study for *in vitro* slow growth was carried out at University of Tuscia, Forest Micro-propagation Lab for *P. alba* (clone 6k3). Research study on Walnut (cv. Sorrento) was carried out at plant biotechnology Lab C.R.A.- FRU, Rome. For *P. alba*, the study was composed of sixteen treatments under light/dark conditions and at 8 °C or 22 °C; the culture medium (a modified MS) was added with different concentration of supplemented with or without the cytokinin 6-Benzylaminopurine (BAP) at two months storage time period. In case of walnut, the research study was composed of eight treatments only under the dark conditions and different concentration of sucrose in culture medium supplemented with or without BAP at 5 °C and 22 °C and at two and three months storage period respectively. In terms of parameters of the study, McKinney Index (injury level %), Survival (%), explants with root formation (%), explants with basal callus formation, average no of explants with vigorous shoots (%) and average number of vigorous axillary shoots per each explant were determined for *Populus alba* (clone 6k3). Regarding the parameters of study in case of walnut, only McKinney index (%) and survival rate (%) were determined.

In terms of the results for poplar with aforementioned parameters, generally most the treatments were recorded to be no significant. Treatment 7 (Dark condition, 60 g/L sucrose and BAP) shows the lowest values for McKinney i.e. (0%) whereas the treatment 14 shows the highest value for McKinney index i.e. (95%). As far as the survival percentage for the poplar explants were concerned, the lowest percentage of survival was recorded for the treatment 14 i.e. (0%) and the highest for treatment 4 i.e. (100%). Similarly in terms of root formation, all the treatments lacking cytokinin in the culture medium stimulates the formation of *in vitro* rooting in poplar. On the contrary, same trend was observed in terms of basal callus formation i.e. all the treatments supplemented with cytokinin forms the basal callus in poplar *in vitro* explants.

Regarding the number of poplar explants with vigorous axillary shoots treatment 9 shows the highest percentage i.e. (80 %). Whereas the highest mean value i.e. (7.66) was recorded for average no vigorous shoots per each explant for the treatment 9 in poplar explants.

Regarding walnut in terms of survival percentages, no significant treatments were recorded during the two months of slow growth storage, but when the results were compared with three months storage period for the same set of treatments, the results were significantly different. Regarding the experiment with walnut cryopreservation, (0 %) survival was recorded in regeneration phase after the treatment of encapsulated dehydrated beads in liquid nitrogen. Hence, further research is suggested for optimal conditions of *in vitro* conservation of *P. alba* (clone 6k3) and *Juglans regia* cv. "Sorrento".

Riassunto

Il presente lavoro è stato dedicato allo studio di due specie legnose: *Juglans regia (*cv. Sorrento) e *Populus alba* (clone 6k3). L'obiettivo principale dello studio è stato quello di sviluppare un protocollo di conservazione in vitro per queste due genotipi/cultivar. Le ricerche sono state condotte presso il laboratorio di Colture in vitro del DIBAF (Università degli Studi della Tuscia) per quanto riguarda il pioppo bianco. Per quanto riguarda il noce, invece, le ricerche sono state condotte presso il Laboratorio di Biotecnologie vegetali del C.R.A. Frutticultura di Roma.

Per quanto riguarda il pioppo bianco lo schema sperimentale è stato articolato in 16 trattamenti in cui gli espianti erano soggetti a condizioni di luce o di buio e a due differenti temperature (8 °C e 22 °C); il terreno di coltura (un MS modificato è stato addizionato con due diversi livelli di saccarosio e si è voluta anche analizzare l'influenza della citochinina 6-Benzylaminopurina (BAP). I trattamenti di crescita rallentata sono stati mantenuti per due mesi.

Nel caso del noce, lo schema sperimentale prevedeva 8 trattamenti esclusivamente al buio a differenti concentrazioni di saccarodio nel terreno di coltura in presenza o meno di BAP , a 5 ° e 22 °C. Il confronto è stato eseguito anche tra un periodo di conservazione di 2 mesi e 3 mesi.

I seguenti parametri sono stati impiegati nello studio del pioppo bianco: l'anali del danno (indice di McKinney), il tasso di sopravvivenza, la formazione dell'apparato radicale (%), la formazione alla base degli espianti di callo, il numero medio di espianti che presentavano germogli ascellari vigorosi (%) ed il numero medio di germogli ascellari vigorosi per espianto. Nel caso del noce sono stati impiegati l'indice di McKinney ed il tasso di sopravvivenza.

Per quanto riguarda il pioppo bianco, in estrema sintesi, l'interazione tra i vari fattori studiati (luce/buio, temperatura, livelli di saccarosio e presenza/assenza di citochinine) ha dimostrato che tutti hanno svolto un effetto significativo sulla sopravvivenza degli espianti e sulla loro capacità di moltiplicazione *in vitro* nella fase di recupero (produzione di germogli ascellari). In generale i trattamenti alla luce hanno evidenziato tassi di sopravvivenza più elevati (tutti superiori al 70%) e danni meno consistenti (al massimo valori intorno al 30%) e la presenza

di BAP ha generalmente influenzato positivamente la conservazione del materiale. Soprattutto in condizioni di buio la presenza di una maggiore quantità di saccarosio ha favorito una migliore conservazione del materiale. In particolare il trattamento che si è contraddistinto contemporaneamente per un livello di danno estremamente basso (1,3 %), un tasso di sopravvivenza molto elevato (86.7 %) e per un numero significativamente più elevato di germogli ascellari prodotti nella fase di recupero (7.6) è stato il numero 9, dove le condizioni sperimentali sono state: 22 °C, luce, presenza di BAP e 30 g/L di saccarosio. D'altro canto, il peggior trattamento in assoluto è stato il n.14 che si è caratterizzato sia per la totale perdita degli espianti (tasso di sopravvivenza pari a 0 %) che per un maggiore indice di danno (95 %); in questo caso le condizioni sperimentali sono state: 22 °C, al buio, terreno di coltura privo di BAP e con 30 g/L di saccarosio.

Per quanto riguarda il noce un prolungamento del periodo di conservazione (3 mesi rispetto a due mesi) ha influenzato negativamente sia la sopravvivenza che ùl'indice di danno.

Introduction

According to the World Conservation Union, approximately over 8000 plants species are facing the threat of extinction. These include both herbaceous and woody plant species. There are several possibilities that can help to save the plant species like, global documentation, distribution and conservation status of the plant species, protection of areas, helping people to understand the values and importance of biodiversity and the existence of national and international legislation etc. (Farnsworth and Sahotra, 2007)

In the last decades, the *ex situ* conservation approach have reached a significant role in the conservation of both threatened as well as economically important woody plant species. The three main principles employed in *ex situ* conservation are: by maintaining living plants in cultivation e.g. botanical gardens, *in vitro* conservation (short and medium term conservation) and cryo conservation (long-term conservation), (Catana et al, 2010).

Slow Growth is one the major tissue culture technique used for the conservation of plant genetic resources in medium term. Growth reduction is generally achieved by amending the culture medium or modifying the environmental conditions. The most widely used technique is temperature reduction, which can be coupled with a decrease in light intensity or culture in dark condition. Modification of the culture medium can include addition of osmotically active compounds, absence or presence of growth regulators, reduction or increase in the sugar concentration and dilution of mineral elements (Engelmann, 2010). For efficient slow growth protocol, it is important to know the ecological and geographical origin of plants. In general, tropical and subtropical plants are less tolerant to the cold temperature than the tropical plants. For example coffee (*Coffea spp*) was successfully preserved at 20°C, while apple germplasam was successfully preserved at 1-4°C. Other parameters that can influence the efficiency of *in-vitro* slow growth protocols are type of explants, their physiological state when entering the storage, culture vessel and its volume (Engelmann, 1991). Storage of *in vitro* meristems or shoot tips has many advantages i.e. low temperatures slow the growth and development in a natural way and reduce the frequency of necessary subcultures. At temperature of 2-5 °C subculture is needed 1-2 times per year. The chances of mutations are lower since these are more linked with the increasing number of subcultures.In addition for commercial micro-propagation labs, it is very important tool to have *in vitro* plant material at any time of the year and slow growth technology at low temperatures ensures this. Similarly

haploid material can be conserved using low temperatures; otherwise it quickly becomes diploid at higher temperatures. (Pierrik, 1987)

Depending on the plant species, researchers have obtained different results by changing the medium formulations, temperature or by addition of osmoticums. Catana and co-workers (2010) while working with conservation of slow growth conditions of two rare plant species from Caryophyllaceae family reported that 10°C and the reducing of the concentration of micro and macro-nutrients at ¼ for *Gypsophila petraea* and at 1/10 for *Dianthus callizonus* allowe the conservation in optimal parameters for more than 12 months. Ozudogru and co-workers (2013) successfully conserved the shoot tips of *Nandina domestica* for six months both at 4°C and 8°C and 60 g/L of sucrose. However, the 4°C treatment, in combination with 60g/L of sucrose, was found to be the best, because less hyperhydicity was observed in *N.domestica* shoots as compared to shoots coming from 8°C storage. Similarly, Lukoseiciute and co-workers (2012) conserved *Fragaria spp* and *Pyrus spp* for 15 and six months, respectively under *in vitro* conditions at 4°C. Wilkins *et al.,* (1988) found that single shoots of *Morus nigra L.* stored on multiplication medium at 4°C with a 16-h photoperiod survived for only six months. They found that this survival could be further increased to 42% at nine months by storing them at 25 °C with activated charcoal. Lundergan and Janick (1979) reported for the first time the successful storage of *Malus domestica* Borkh "Golden Delicious" for 12 months at 1°C or 4°C. Orlikowska (1991, 1992) reported that two apple rootstock cultivars kept at 4°C, in dark, in a medium containing BAP stored better than in the medium lacking growth regulator. Sharma and Thorpe (1990) reported successful storage of 15 genotypes of *Morus alba* L. at 4°C in dark on shoot proliferation medium with high viability (80%) for six months. All shoots rooted and surviving shoots retained their multiplication potential. Capuana and Leonardo (2013), while working with the slow growth strategies for *Castanea sativa,* reported 82% survival of explants over slow growth duration of 48 months at 8°C. At storage temperature of 4°C, the survival of shoots was significantly lower than at higher temperature with approximately 56% surviving after 12 months, and no plants recovered after 24-month storage. They observed that the addition of 0.44 μM of BAP in storage medium has positive effect on recovery of healthy shoots during the recovery phase while the addition of abscisic acid did not significantly affect the survival of shoots following the storage. A low amount of light was also found to have positive effects on the rate of shoot survival after storage period.

Cryopreservation is the conservation of living plant propagules at very low temperature (-196C). It involves a viable and economical method for the long-term conservation of genetic resources of vegetatively propagated plants. A number of techniques have been developed to minimize desiccation and freezing damage and, to ensure high propagule recovery after cryo treatment. The most preferred material used for cryo-preservation is shoot apices from *in vitro-* grown shoots of vegetatively propagated plants. Broadly speaking, there are two main kinds of cryopreservation techniques classical methods (freeze induced dehydration) and new methods (vitrification of internal solutions). There are already some cryo banks around the world. Cryopreserved collections of coffee seeds are being established in Tropical Agricultural Research and Higher Education Center (CATIE; Cañas, Guanacaste, Costa Rica) and in IRD (Montpellier, France), using a protocol including controlled dehydration and freezing (Desserts and Engelmann 2006). In the case of dormant buds, the 2,200 accessions of the US apple germplasm field collection are duplicated under Cryopreservation (Forsline et al. 1999), as is the case for the 420 accessions of the mulberry field collection maintained at the National Institute of Aerobiological Resources (Yamagata, Japan; Niino 1995). Both *in vitro* and cryo-preservation can solve the problem of maintaining the economically and socially important woody plant species for future uses in both medium term and long-term programs especially for breeding purposes.

It is important to study *in vitro* conservation of poplar and walnut because both have got considerable economic importance. Poplars and their hybrids are the fastest growing trees in the temperate zone with mean annual increments of above-ground biomass approaching 14 dry metric tons per hectare per year culminating in less than ten years as measured in today's commercial plantations (Stanton, 2007). Similarly poplar trees were among the first plants to be stably transformed (Fillatti et al, 1987). Regarding walnut importance, it has dual uses (i.e. nuts and wood products). Nuts are used for human consumption in many different ways either consumed directly or used in different dietary products. The American Indians used the inner bark tea as an emetic and laxative. The bark is still used as dentifrice in Pakistan. Moreover the juglone from fruit and bark of *J. nigra* acts against dermatomycosis. Ellagic acid and juglone from walnut are being also studied for possible use as a cancer therapy drugs. The walnut wood is of very high quality and is used to make furniture, veneers, gunstocks etc. (Virginia tech, 2004).

Juglans has proven to be a genus that is also hard to be propagating by tissue culture as well as by vegetative propagation. Some problems relating to tissue culture of walnut are: internal contamination of plant material and susceptibility of explants to damage caused by disinfectant substances, oxidation of the explants i.e. phenolic compounds, low maladaptability to the culture media, poor proliferation, poor elongation and rooting rates, difficult acclimatization of the rooted propagules and complications when growing small plantlets in the nursery (Lopez, 2004).

Present study aimed to establish effective protocol for *in vitro* cold storage of *P. alba* and *J. regia in vitro* explants in order to reduce the growth and increase the subculture time interval in medium term. Since maintaining cultures at standard growth chamber conditions requires frequent subcultures and, ultimately, increases the costs of lab. Similarly, increasing the number of multiplication cycles may lead to changes in the juvenility/maturity characters, including rooting and flowering behavior (Hausman *et al*, 1994).

2. Conservation of Plant Genetic Resources

Plant genetic resources for food and agriculture are the main bases of global food security for the future. They represent diversity of genetic material retained in local varieties, modern cultivars, crop wild relatives and other undomesticated species. Genetic diversity provides opportunities for growers and plant breeders with possibility to develop, through selection and breeding, new and more productive crops that are more resistant to virulent pests and diseases and adapted to changing environments. The world population is estimated to reach eight billion by the year 2020 and food cereals production will have to be doubled from the present level of about five billion tons per year. To meet the demand for more food, it will be necessary to make better use presently available world's wild plant genetic diversity. Still, plant genetic resources are disappearing at alarmingly fast rates. The causes for these losses are numerous and include extensive deforestation in developing countries. Other factors are construction of hydroelectric projects, road laying, urbanization and changes in agricultural practices, and lastly modern agriculture and introduction of new and uniform varieties. More than 15 million hectares of tropical forests are squandered each year for illegal logging. Genetically uniform modern varieties are substituting the highly unique local cultivars and landraces in traditional agro ecosystems. Over-grazing by animals and changes in land-use pattern are taking heavy impact on diversity available in the wild species. Urbanization and changing life styles patterns, globalization and capitalistic economies are also contributing indirectly to the loss of genetic diversity, particularly of minor and neglected crops. Such losses will have serious implications for food security in the long term.

Global outcry about loss of valuable genetic resources incites international action. Programs for preservation of plant genetic resources for food and agriculture were thus started and gene banks were established in many countries. The main objective was to accumulate and maintain the genetic diversity in order to ensure its continued availability to meet the needs of different users. The logic of germplasm conservation demands that, acquiring methods, initially capture maximum variation and later on, conservation and regeneration techniques that can help in minimizing losses through time (Astley, 1992). To this effect, plant genetic resources (PGR) conservation activities are composed of collecting, conservation and management, sorting out of potentially valuable plant material by characterization and evaluation for afterwards use. Recent progress in biotechnology, especially in the area of *in*

vitro culture techniques and molecular biology supply some important tools for better conservation and management of plant genetic resources (Rao 2004).

2.1 *In-situ* Conservation

In situ conservation involves a set of safeguarding techniques including the designation, management and monitoring of biodiversity in the same area where it is originated. *In situ* management involves two strategies i.e. species centered approach or whole ecosystem based approach. In the former approach only conservation of targeted populations of selected species are involved at their natural habitat. In the latter case, the whole ecosystem is protected. (Lacy 2010).

2.2 *Ex- situ* Conservation Through plant tissue culture Technology

Tissue culture techniques are of great importance and interest for the collection, multiplication and storage of plant germplasm. A tissue culture system allows propagating plant material with high multiplication rates in a relatively short period of time in an aseptic environment. Virus free plants can be obtained through meristem culture in combination with thermotherapy, thus helping in the production of the diseases free plant stocks and facilitating the quarantine measures for the international exchange of germplasam. Also due to the reduce size plants, allows in reducing the space requirements and consequently lab costs for the maintenance of the germplasam collections. So far, *in vitro* propagation protocols for several thousand species have been successful established, including numerous rare and endangered species. (Engelmann 2010)

In parallel to *in vitro-* propagation, different *in vitro* conservation methods are employed for the conservation of plants genetic resources either in medium term or for longer period of time. For short and medium term storage, the aim is to reduce growth and to increase the intervals between the subcultures. For long term storage, cryopreservation, i.e. storage at ultra −low temperature, the only current method in use is the conservation in liquid nitrogen (-196C), (Engelman 2010).

2.2.1 *In vitro* conservation by slow growth

In slow-growth *in-vitro* conservation germoplasms are cultured under growth limiting conditions either by the use of only cold temperature or osmoticums or both.

In case of osmotics, such as sugars, there effect is to reduce the water potential of the plant cells. It has been found that the addition of the osmotics to the culture medium reduces the growth of the culture and increases the storage life of many of the *in vitro* growing tissues of different plant species. Keeping in the mind, the hypothesis that the turgor drives growth and cell expansion, high levels of osmotic agents can act against the creation of critical turgor pressure, which must be established before cell expansion can occur. Due to this stress condition, both shoot formation and callus formation will be inhibited.

Common osmotics used for *in vitro* studies are mannitol, sucrose, and sorbitol. Tributyl 1-2, 4 diclorobenzlyphosphonium chloride (Phosphon D), malic hydrazide, succinic acid-2, 2-dimethyl hydazide (B-995), CCC and ancymidole were also found to be good chemicals for the prolongation of the storage life of the *in vitro* grown tissues. Sucrose is the major source of the carbon in most of the tissue culture media and it can be used to reduce the plant *in vitro* growth acting on its concentration. (Sarkar and Nail, 1998). In case of potato and pear micro-plants, elongation was decreased with the increasing concentration of the sucrose (Tahtamouni and Shibili 1999). For the tobacco callus cultures, 30 g/L were found to affect the capacity of cultures to form shoots and this ability was completely inhibited when the sucrose reached to level of 150 g/L. similarly, mannitol, a sugar alcohol, which is the primary photosynthetic product in some plants can also be used as osmotic agent. The use of mannitol suppresses the tissues growth in chrysanthemum (Shibli *et al*, 2003) and bitter almond (Vieitez *et al*, 1997), but do not have any significant effect on the tissue growth in tobacco. Sorbitol is another sugar alcohol used for the *in vitro* preservation experiments. Shibli and co-workers (2003) observed that at high sucrose, sorbitol or mannitol concentration significantly reduced the growth of bitter almond micro-shoots and extended the subcultures intervals to four months when cultures were kept at room temperature.

Sometimes cultures can be preserved without any addition of osmoticums, only by putting the explants at temperature just above freezing. Under such conditions the accumulation of

unsaturated lipids in the cell membrane would cause cell membrane thickenings and retard the cell division and elongation.

Light is also an important factor for slow growth preservation of germplasam. Sometime cultures are stored either in complete darkness or under very low light conditions at low temperatures with the modification of culture medium.

The main objective of the slow growth *in vitro* conservation of germplasm is to reduce the number of frequency of the subcultures and to maintain the genetic diversity of the species in sterile conditions without compromising plant genetic stability.

2.2.2 Cryopreservation

Cryopreservation is the conservation at very low temperature (− 196ºC) of living propagules. Cryopreservation offers a viable and economical method for the long-term conservation of genetic resources of vegetatively propagated plants. Various techniques have been developed to minimize desiccation and freezing damage, thus ensuring high propagule recovery. In most cases, shoot apices obtained from *in vitro*-grown shoots are the plant material used for cryopreservation of vegetatively propagated plants. Cryopreservation techniques are based on either freeze-induced dehydration (classical methods), or vitrification of internal solutions (new methods). Various considerations should be taken into account when cryopreservation techniques are used for germplasm conservation. (Benito *et al*. 2004)

2.2.3 Classical cryopreservation Techniques

The term classical cryoconservation involves the stepwise gradual slow cooling of the plant tissues to a defined pre-freezing temperature, followed by the rapid immersion in the liquid nitrogen. With temperature reduction during slow cooling, the cells and the external medium initially supercool, followed by ice formation in the medium (Mazur, 1984). The cell membrane acts as a physical barrier and prevents the ice from seeding the cell interior, and the cells remain unfrozen but super cooled. As the temperature is further decreased, an increasing amount of the extracellular solution is converted into ice, thus resulting in the concentration of intracellular solutes. Since cells remain super cooled and their aqueous vapor pressure exceeds that of the frozen external compartment, cells equilibrate by loss of water to external ice. Depending upon the rate of cooling and the pre-freezing temperature,

different amounts of water will leave the cell before the intracellular contents solidify. In optimal conditions, most or all intracellular freezable water is removed, thus reducing or avoiding detrimental intracellular ice formation upon subsequent immersion of the specimen in liquid nitrogen. However, too intense freeze-induced dehydration can incur different damaging events due to concentration of intracellular salts and changes in the cell membrane (Meryman et al, 1977). Rewarming should be as rapid as possible to avoid the phenomenon of recrystallization in which ice melts and reforms at a thermodynamically favourable, larger and more damaging crystal size (Mazur, 1984).

Classical freezing procedures include the following successive steps: pre-growth of samples, cryoprotection, slow cooling (0.5–2.0°C/min) to a determined prefreezing temperature (usually around −40°C), and rapid immersion of samples in liquid nitrogen, storage, rapid thawing and recovery. Classical techniques are generally operationally complex since they require the use of sophisticated and expensive programmable freezers. In some cases, their use can be avoided by performing the slow-freezing step with a domestic or laboratory freezer (Kartha and Engelmann, 1994).

Classical cryopreservation techniques have been successfully applied to undifferentiated culture systems such as cell suspensions and calluses (Kartha and Engelmann 1994; Withers and Engelmann 1998) and apices of cold-tolerant species (Reed and Uchendu, 2008).

2.2.4 Advanced Cryopreservation Techniques

In vitrification-based procedures, cell dehydration is carried out prior to freezing by exposure of living tissues to concentrated cryoprotective media and/or air desiccation and by subsequently rapid cooling. As result, all factors that induce intracellular ice formation are avoided. Glass transitions (modification in the morphological conformation of the glass) have been noted with numerous materials using thermal analysis (Sakai et al. 1990; Dereuddre et al. 1991; Niino et al, 1992). Vitrification-based pathways render practical advantages in comparison to standard classical freezing techniques. They are more desirable for complex organs (shoot tips, embryos), which are composed of variety of cell types, each with specific requirements. By preventing ice formation in the system, vitrification-based procedures are practically less complex than classical ones (e.g. they do not require the use of controlled

freezers) and have greater utility for broad applicability, demanding only minor modifications for different cell types (Engelmann 1997).

A common aspect to all these new protocols is that the crucial step to achieve survival is the dehydration step and not the freezing step, as in classical freezing protocols. Hence, if tissues or organs to be conserved are amenable to desiccated down to comparably low water contents (which fluctuates depending on the techniques applied and the type and characteristics of the propagule to be frozen) with no further drop in survival is generally seen after cryopreservation (Engelmann 1997).

The vitrification-based methods can be identified mainly as: (a) encapsulation–dehydration, (b) vitrification (c) encapsulation–vitrification, (d) dehydration, (e) pregrowth, (f) pregrowth–dehydration and (g) droplet–vitrification.

The encapsulation–dehydration practice is centered on the technology developed for the production of artificial seeds. Explants are included in alginate beads, cultured in liquid medium supplemented with sucrose for 1 to 7 d, fractionally desiccated in the air current of a laminar air flow cabinet or with silica gel to a water content around 20% (fresh weight basis), then frozen rapidly. Survival percentage is high and re-growth of cryopreserved samples is generally fast and direct, without callus formation. This approach has been applied to apices of many species from temperate and of tropical descent as well as to cell suspensions and somatic embryos of several species (Gonzalez-Arnao and Engelmann2006; Engelmann *et al.* 2008).

Vitrification involves treatment of samples with cryoprotective substances (highly concentrated vitrification solutions) rapid cooling and rewarming, removal of cryoprotectants and recovery. This protocol has been successfully applied to shoot apices, cell suspensions and somatic cells of numerous species (Sakai and Engelmann 2007; Sakai *et al*. 2008).

Encapsulation–vitrification is a combination of encapsulation– dehydration and vitrification procedures, in which samples are encapsulated in alginate beads, and then treated with the vitrification solutions. It has been employed to shoot apices of several species (Sakai and Engelmann 2007; Sakai *et al*, 2008). Dehydration is the simplest procedure since it consists

of dehydrating explants, then freezing them rapidly by direct immersion in liquid nitrogen. This technique is chiefly used with zygotic embryos or embryonic axes separated from seeds. It has been applied to embryos of a large number of recalcitrant and intermediate species (Engelmann 1997).

Desiccation is usually performed in the air current of a laminar airflow cabinet, but more precise and reproducible dehydration conditions are achieved by using a flow of sterile compressed air or silica gel. Ultra-rapid drying in a stream of compressed dry air (a process called flash drying developed by Berea's group in South Africa) allows freezing samples with a relatively high water content, thus reducing the risks of desiccation injury (Berjak *et al*. 1989). Optimal survival is generally obtained when samples are frozen with a water content comprised between 10% and 20% (fresh weight basis).

The pregrowth technique consists of cultivating samples in the presence of cryoprotectants, then freezing them rapidly by direct immersion in liquid nitrogen. This technique has been successfully developed for *Musa* meristematic cultures (Panis *et al.* 2002).

In a pregrowth–dehydration procedure, explants are pregrown in the presence of cryoprotectants, dehydrated under the laminar airflow cabinet or with silica gel and then frozen rapidly. This technique has been applied distinctly to asparagus stem segments, oil palm somatic embryos and coconut zygotic embryos (Uragami *et al.* 1990; Assy-bah and Engelmann 1992; Dumet *et al.* 1993).

Droplet–vitrification is the latest technique developed (Sakai and Engelmann 2007). The number of species to which it has been successfully applied is increasing steadily. Apices are pretreated with vitrification solution, then placed on an aluminum foil in minute droplets of vitrification solution and frozen rapidly in liquid nitrogen.

2.2.5 Importance of *in vitro* and cryopreservation

Despite the fact that its routine use is still limited, there are an expanding number of gene banks and botanic gardens where cryopreservation is in work on a large scale for different types of materials, which are, or are not, tolerant to desiccation.

In the case of conventional seed species, cryopreservation is used mainly for storing seeds with limited life span and of rare unique or endangered species while propagules are used for species that have to be vegetatively propagated. The National Center for Genetic Resources Preservation (NCGRP; Fort Collins, CO) protects 43,400 plant accessions over the vapors of liquid nitrogen (Walters 2010). The National Bureau for Plant Genetic Resources (NBPGR; New Delhi, India) stores 1,200 accessions from 50 various species, consisting mainly of threatened medicinal plants (Mandal, 2000). Cryopreservation is also applied to seeds which are permissive to freezing. Cryopreserved stockpile of coffee seeds are being established in Tropical Agricultural Research and Higher Education Center (CATIE; Cañas, Guanacaste, Costa Rica) and in IRD (Montpellier, France), using a protocol including controlled dehydration and freezing (Dussert and Engelmann 2006).

In the case of dormant buds, the 2,200 accessions of the US apple germplasm field collection are copied under Cryopreservation (Forsline *et al.* 1999), as is the case of the 420 accessions of the mulberry field collection maintained at the National Institute of Aerobiological Resources (Yamagata, Japan; Niino 1995). Inactive buds of more than 440 European elm accessions are conserved in liquid nitrogen by Afocel (Bordeaux Nangis, France; Harvengt *et al.* 2004), and research is under way in France (IRD) and the USA (NCGRP) for vitis germplasm preservation.

Finally, cryopreservation is being employed in gene banks for long-time period storage of genetic resources of vegetatively proliferating species, using shoot apices collected from *in vitro* plants. The plant for which the development of cryo-preservation protocols is most advanced is potato, since more than 1,000 old potato varieties are cryostored in Gatersleben, Germany at the Leibnitz Institute of Plant Genetics and Crop Plant Research (Keller et al. 2005, 2006) and more than 200 accessions at the International Potato Center (Lima, Peru; Golmirzaie and Panta 2000). A copy of nearly 100 accessions of the *Pyrus* field collection National Clonal Germplasm Repository (NCGR; Corvallis, OR) is cryostored at NCGR, with another duplicate at the NCGRP (Reed et al. 2000). In Korea, two cryopreserved groups of Allium have been setup, which constitute an aggregate of more than 800 accessions (Kim *et al*. 2009).

2.2.6 Recent Uses of Cryopreservation

Recently, cryopreservation has been used for cryotherapy, i.e. for eliminating viruses from infected plants, as a substitute or in complement to classical virus eradication techniques such as meristem culture and cryotherapy (Wang et al. 2008). In cryotherapy, plant pathogens such as viruses, phytoplasmas and bacteria are abolished from shoot tips by introducing them briefly into liquid nitrogen. Unequal dispersion of viruses and obligate vasculature-limited bacteria in shoot tips allows eradication of the infected cells by injuring them with the cryotreatment and reestablishing of alive healthy shoots from the surviving pathogen-free meristematic cells. Thermotherapy followed by cryotherapy of shoot tips can be used to enhance virus eradication. Cryotherapy of shoot tips is a flexible procedure to carry out. It permits treatment of large numbers of sampling material and results in a high recurrence of pathogen-free regenerants. Difficulties related to excision and regeneration of small meristems is circumvented. To date, acute pathogens in banana (*Musa spp*.), *Citrus spp*., grapevine (*Vitis vinifera*), *Prunus spp*., raspberry (*Rubus idaeus*), potato (*Solanum tuberosum*) and sweet potato (*Ipomoea batatas*) have been eliminated using cryotherapy. These organisms comprises nine viruses (banana streak virus, cucumber mosaic virus, grapevine virus A, plumpox virus, potato leaf roll virus, potato virus Y, raspberry bushy dwarf virus, sweet potato feathery mottle virus and sweet potato chlorotic stunt virus), a sweet potato little leaf phytoplasma and a bacterium, Huanglongbing.

3. Genus *Populus*

3.1 Biology of *Populus*

 Species of the genus *Populus* (frequently known as aspens, cottonwoods, and poplars) are deciduous or, hardly semi-evergreen trees that arise chiefly in the boreal, temperate, and subtropical belts of the northern hemisphere (Eckenwalder, 1996; Dickmann, 2001; Cronk, 2005). Trees from this genus normally have tall and upright single main stems, with bark that tends to remain thin and smooth until more late ages than in other species (Eckenwalder, 1996; Dickmann, 2001).They seldom live longer than 100–200 years, but are included in the group of fastest growing temperate trees and can arrive at large sizes. A distinguished case is black cottonwood(*Populus trichocarpa*), which can go beyond 60 m in height and reach up to 3 m in width (DeBell, 1990).

White Poplar is a fast-growing, deciduous tree of temperate region, which can reach up to 60 till 100 feet in height with a 40 to50-foot-spread and makes a comfortable shade tree, albeit it is considered short-lived. The dark green and lobed leaves have a flossy, white underside which gives the tree a unique sparkling effect when breezes stir through the leaves. The *P. alba* leaves are entirely covered with these white curly hairs when they are young and first open. The fall colors of the leaves are pale yellow. The flowers come in to appearance before the leaves in spring but are not showy, and are subsequently followed by small, fuzzy seedpods which contain many seeds. It is the white stem and bark of white poplar which is distinctly dazzling, along with the beautiful two-toned leaves. The bark stays uniform and white until very old when it can become ridged and furrowed. The wood of white poplar is somewhat brittle and can be subject to damage in storms. Leaves starts to falling from the trees beginning in summer and continue dropping till the fall.

3.2 Habitat

Most species of *Populus* have wide native ranges, often spanning more than 20 degrees of latitude and a great diversity of climates and soils (Eckenwalder, 1996; Dickmann, 2001). *Populus* trees grow in a striking variety of habitats, ranging from hot and arid, desert-like sites in central Asia and northern and central Africa to alpine or boreal forests in Europe and North America. They are shade and drought-intolerant and seed establishment typically depends on major disturbances, such as fire, floods, or ice scours (Romme et al., 1995, 1997; Rood et al., 2007).

Poplar prefers soils with a sandy loam texture. Poplars are hygrophilous in general and very sensitive to drought stress. They grow very well in soils with standing water or prolonged submersion during the growing season. Poplar is generally thermophilous species, having good tolerance to high temperatures. Tolerance to early frost depends on the latitude of the provenance. Poplar species are known to colonize areas with the bare mineral soil such as those arise in riparian areas after flooding events. After the flooding events poplar seeds germinate after few hours and after 2 days the cotyledons are visible. Poplars have very good capability to sprout new shoots from the portions of branches and stems transported by floods and cover in the soil and they also have also very good capability to form root suckers.

Photos from Paglia River, Poplar spp (Field visit with Prof. Maurizio Sabatti, September 2013)

3.3 Economic and Environmental Importance of Poplar spp.

A poplar is a forest tree species of major economic and environmental significance for many countries around the world. They have unique ability to adapt to wide range of climatic and soil conditions, from extreme dessert heat to strong mountain winds. It is easy to perform agronomic practices with poplars and they are the most important species nowdays in agroforestry systems, especially for small scale farmers. It provides a great variety of wood products (such as plywood, veneer, industrial round wood, pallets and furniture), non-wood products (fodder, fuel wood) and services (shelter, shade, and protection of soil, water, crops live-stalk and dwellings), (Garyfallos Arabatzis, 2008).

The wood is diffuse-porous, light in weight and yet capable of building trees of 40 m height in less than 20 years. Several of these features have made poplars attractive to humans since ancient times. Today, poplars are cultivated worldwide in plantations for pulp and paper, veneer, excelsior (packing material), engineered wood products (*e.g*., oriented strand board), lumber, and energy. Grown at a commercial scale under intensive culture for 6-8 year rotations, production rates with hybrid poplar can be as high as 17-30 Mg/ha/yr of dry woody biomass (Zsuffa *et al*. 1996), comparable to the biomass produced by row crops such as corn. Historically, poplars have been widely used in windbreaks and for erosion control. Most recently, poplars have proven to be effective in the phytoremediation of environmental toxins (Flathman and Lanza 1998) and as bio indicators for ozone pollution in the environment (Jepsen 1994).

According to global reported data planted poplar stands accounts for 6.67 million hectares, of which 3.8 million ha (56%) were planted primarily for wood production and 2.9 million ha for environmental purposes. Of the total reported plated area of poplars, thirty percent of planted area was established in agroforestry systems, which also accounted for 40% of global poplar wood production. Taking a look at the global poplar map, we observed that the 73% of the world total poplar plantations are present in China. These plantations are attributed for 53% of the global plantations used for the wood production and almost all of those planted for environmental purposes. Similarly poplar plantations established for the agro-forestry systems, 49% are located in China and equal percentage in India. Countries like (Turkey,

China, France, India and Italy) annually harvest more than 1 million cubic meters of poplar wood from agro-forestry systems (ball *et al,* 2005)

White poplars have also be shown to have the potential to be used as biomonitor of trace of metals elements like (As, Cd, Cu, Fe, Mn, Ni, Pb and Zn) in leaves and stems of white poplar (*Populus alba*) trees . Madejon et al. (2004) selected 25 trees in the riparian forest of the Guadiamar River (S. Spain); one year after this area was contaminated by a mine spill, and 10 trees in non-affected sites. The spill-affected soils had significantly higher levels of available cadmium (mean of 1.25 mg kg^{-1}), zinc (117 mg kg^{-1}), lead (63.3 mg kg^{-1}), copper (58.0 mg kg^{-1}) and arsenic (1.70 mg kg^{-1}), than non-affected sites. They founded that the concentration of trace element in poplar leaves was positively and significantly correlated with the soil presence of cadmium and zinc, and to a lesser extent for arsenic. Thus, poplar leaves could be used as biomonitors for soil pollution of Cd and Zn, and moderately for As. (Madejon et al., 2004)

3.4 Poplar as Model Plant

The necessity for model plant species has been well recognized to understand the different process in plants in depth. For this reason, Arabidopsis has gain supreme acceptance among the plant scientists. The reason of Arabidopsis being so popular among scientific community is that it is small plant with small genome and rapid life cycle. Also it can be very easily transformed and has wide natural distribution. But it has debated among plant scientists that Arabidopsis cannot be a useful model plant for crops such as rice, wheat and maize, because these agronomic crops are mono-cotyledons. Due this, the physiology, biochemistry and development of these species may not be adequately studied in a dicotyledonous weed. Similarly the other important question arises that if Arabidopsis can act as model plant for trees species. Tree species are very complex in nature as compared to non-woody plants. The most obvious manifestation of this is the development of wood or secondary xylem from the vascular cambium. In addition, to secondary xylem formation, trees also shows the complex pattern of the activity and control of, for example, bud-burst must involve a complex interactions between environmental signals (including day length and temperature) and plant signals transduction pathways. For a plant like Arabidopsis it is hard to imagine that it could be adequate for the study processes in their entirety, since control may not be only at the level of the gene expression. Similarly the age at which different tree species flower also varies greatly. For example in case of Salix it takes one year, poplar six years and quercus 60 years after germination. In order to better understand further some of the unique processes that occur in woody plants and which are not present in Arabidopsis, such dormancy and secondary wood formation, a model tree was needed. The reason that poplar was chosen as model tree were the relatively small genome size (450-550 Mbp), the large molecular genetic maps and the ease of genetic transformation. Also poplar can be easily propagated vegetatively means, meaning a sufficient amount of experimental materials for trials. (Gail Taylor, 2002).

4. Genus *Juglans*

The genus *juglans* includes 15 species splitted into three groups namely, common walnut (*Juglans regia*), black walnut (*Juglans nigra*, *Juglans hindsii*) and grey and white walnuts (of low interest).

4.1 Biology

J. regia which is also known as (Common walnut, Persian walnut or English walnut), is the true walnut tree of the Old World. It is native in a region ranging from the balkans eastward to the Himalayas and southwest China. *J. regia* is a huge deciduous tree, gaining heights of about 25–35 m, and the trunk can grow to 2 m in diameter. The main trunk is commonly short with a broad crown, though may be taller and narrower than usually observed in managed plantations, as compared in dense forest competition. It is a light-loving species, requiring full sun to grow well. The bark is soft and smooth, greenish-brown when young and silvery-grey on older branches, with unevenly distributed broad fissures with a rough texture. Like all *Juglans* species, the pith of the twigs has air spaces. The *J. regia* tree leaves are alternately arranged, 25-40 cm long, odd-pinnate with 5–9 leaflets, paired alternately with one terminal leaflet (Verma et al, 2009). Walnut is monoecious flowering tree and has unisexual flowers on same tree. The flowering time is from April to May. The flowering behavior is proteroandry, means that male flowers open and mature before female flowers. The flowers may bloom in solitary or in groups of 2-3 at the apex of the shoots of the year in combination with the leaves. The pollination is anemophilous. The walnut fruit is drupe that is ready to harvest in late September. It consists of hull which combines together epicarp and mesocarp and a woody endocarp containing the fruit, also called kernels.

4.2 Habitat

The common walnut is a heliophilous species and moderately thermophilious. It is resistant to low temperature in winter, but can be damaged by the late frost. *J. regia* requirement for cold is about 1000-1500 hours at 7ºC. It requires rainfall of around 800-900 mm, well distributed along the growing season. It is sensitive to the periods of summer droughts and to the stations with strong winds. Walnut prefers good site quality located in the plain, in the hills and foothills.

4.3 Economic Importance of *Juglans Regia*

Walnut has been used globally by human beings since ancient times. The wood of walnut has got very good quality to be used in different articles. The walnut kernel is extensively used in oil well drilling for lost circulation material in making and maintaining seals in fracture zones and unconsolidated formations. Walnut shells have also been used in paints industry. The paint and varnishes mixed walnut shells are far superior quality than the ordinary sand paint. The walnut shells are also used in the soap, cosmetics, and dental cleansers as rough agent. The explosive industry uses the walnut kernels as filler in dynamite. The walnut shell has also very important use in metal cleaning and polishing. It is used for cleaning the jet engines, electronic circuit boards, ships and automobile gear systems (Virginia tech, 2001)

5. Materials and Methods

5.1 Plant Material

For our *in vitro* slow growth experiments we use *Populus alba* (clone 6k3) which is originally coming from the northern Italy that was previously evaluated in common garden studies at the University of Tuscia. Generally it is believed that clone 6k3 is cold tolerant, as it is native to cold area. In case of walnut, *in vitro* grown plantlets of a seedling of the cultivar "Sorrento" were used in the experiments. This seedling is currently undergoing the evaluation trials at CRA-FRU, Rome, as the root-stock for the *Juglans regia* cultivars.

5.2 Establishment of Tissue culture for Poplar and Walnut

To perform the experiments for slow growth with Poplar and Walnut, one month old *in vitro* plants from multiplication cultures were used. In case of poplar, MS (Murashige and Skoog (1961) modified according to Lubrano (1992) was used and supplemented with or without BAP. For walnut DKW medium (Driver and Kuniuki, 1984) was used. Also the DKW medium was supplemented with or without BAP.

In case of poplar, *in vitro* rooted material was used in slow growth experiments. Before placing poplar explants into slow growth conditions, the roots were excised on clean sterile petri dishes. Individual shoots were then cultured on the storage medium. For walnut cluster of shoots, coming from the multiplication cultures were used.

5.3 Culture Conditions

The cultures were put in growth chamber at 8 °C and 22°C in case of poplar both at light(16h photoperiod) and under the dark conditions, while in case of walnut the cultures were incubated at 5°C or 22°C in refrigerator under the darkness.

5.4 Post Slow Growth Evaluation

To test the effect of the different treatments in terms of survival and injury level and other parameters of the single explants and to monitorate their general condition we tested the following parameters

Poplar

a) Survival percentage (at the third subculturing during regeneration)
b) McKinney Index (after two months of slow growth period) (McKinney, 1923)
c) Root formation percentage (after two months of slow growth period)
d) No of plants with callus formation (after two months of slow growth period)
e) Percentage of plants with vigorous axillary shoots per explant (at the third sub culturing period during the slow growth)

f) Average no of vigorous axillary shoots per explants (at the third sub culturing period during the slow growth)
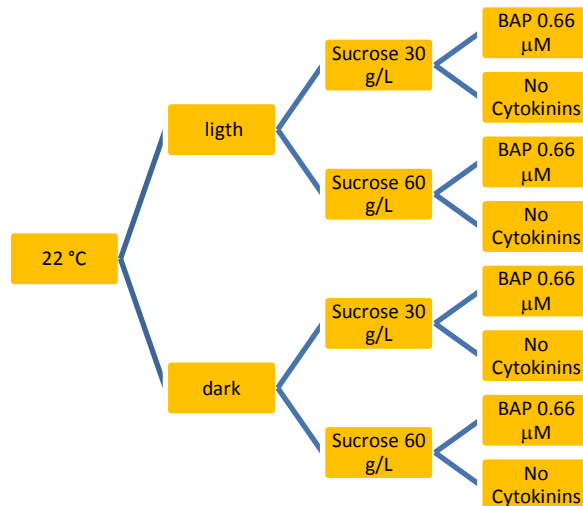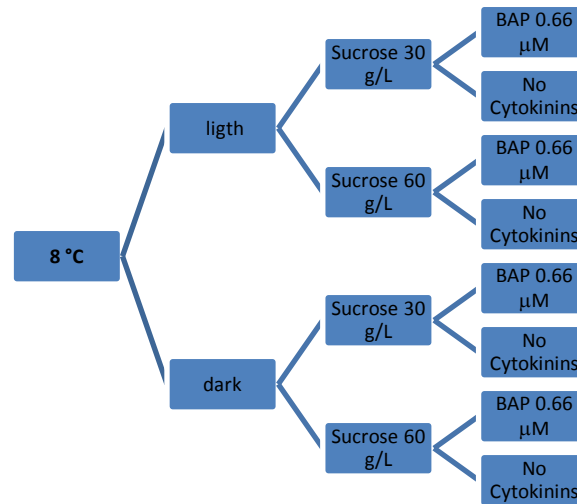
Walnut

a) Survival Percentage (after 2 and 3 months of cold storage)
b) McKinney index (after 2 and 3 months of cold storage) (McKinney, 1923)

The calculations for the above parameters are described below in detail

a) Survival percentage obtained by means of the percentage ratio between the explants that appeared viable and the initial explants (n.o of viable explants per vessel/ n.o of initial explants *100).

b) McKinney Index (RIF) obtained by the means of the percentage ratio between the explants that appeared damage, according to the classes of damage previously established and the initial explants (no of damaged explants per vessel /n.o of initial explants *100)

c) Root formation percentage obtained by means of the percentage ratio between the explants that formed roots and the initial explants (n.o of rooted explants per vessel/ n.o of initial explants *100).

d) Basal callus formation percentage obtained by means of the percentage ratio between the explants that formed basal callus and the initial explants (n.o of explants with basal callus per vessel/ n.o of initial explants *100).

e) Percentage of explants with vigorous axillary shoots were obtained by means of ratio between the explants that formed more than two shoots per explant and the initial explants (n.o of explants with more than two shoots per each explant / n.o of initial explants *100).

f) Average no of vigorous axillary shoots per explant were obtained by means of ratio between the explants that formed vigorous axillary shoots per explant and the initial explants (average no. of vigorous axillary shoots per explant / n.o of initial explants *100).

5.5 Experimental Design

Populus alba genotype 6K3 in MS media modified by Lubrano (1992) - 2 month of cold storage – 15 explants for each treatment (5 explants per vessel) explant = single stem coming from *in vitro* rooted plants

8 °C
- ligth
  - Sucrose 30 g/L
    - BAP 0.66 µM
    - No Cytokinins
  - Sucrose 60 g/L
    - BAP 0.66 µM
    - No Cytokinins
- dark
  - Sucrose 30 g/L
    - BAP 0.66 µM
    - No Cytokinins
  - Sucrose 60 g/L
    - BAP 0.66 µM
    - No Cytokinins

22 °C
- ligth
  - Sucrose 30 g/L
    - BAP 0.66 µM
    - No Cytokinins
  - Sucrose 60 g/L
    - BAP 0.66 µM
    - No Cytokinins
- dark
  - Sucrose 30 g/L
    - BAP 0.66 µM
    - No Cytokinins
  - Sucrose 60 g/L
    - BAP 0.66 µM
    - No Cytokinins

5.6 Experimental Design for Walnut

Juglans regia,sorrento seedlings in DKW medium
2 month or 3 months of cold storage – 15 explants for each treatment (5
explants per vessel)
explant = shoot cluster coming from *in vitro* shoot cultures
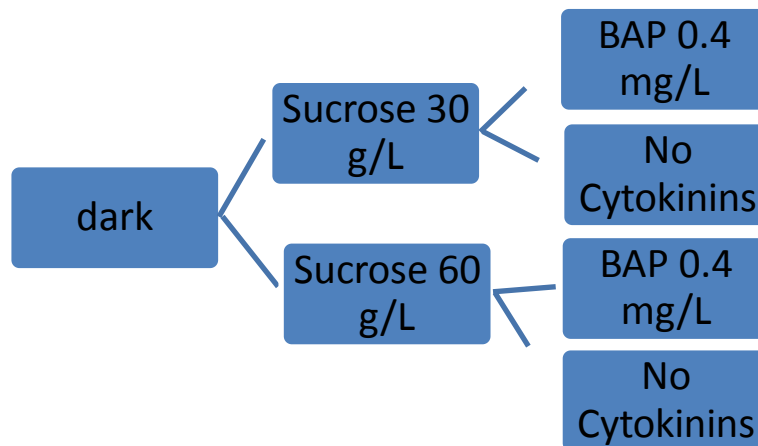
5.7 Statistical analysis

The data was analyzed by software Stata 11.0 by performing Anova and Post-hoc analysis (Fischer's Least Significant Difference (LSD); $P < 0.05$).

6. Results and Discussion

In this section, the results and discussion regarding *in vitro* storage experiments regarding *P. alba* and *J.regia* are presented. For Poplar sixteen experiments were carried out at 8 °C and 22°C with 30 or 60g/L of sucrose under light and dark conditions.

While in case of walnut, only the effect of 5°C or 22°C under conditions in the medium supplemented with 30g or 60 g/L of sucrose, in presence or absence of BAP in darkness condition were analyzed.

6.1 *Populus alba*

The 6K3 genotype of *P. alba* used in the present study was no tested before in a slow growth condition experiment. The micropropagation of *P. alba* is a very high time-consuming practice because it must be subcultured each 10-12 days. So, even if the result in this area often depends from the genotype, preliminary information on the suitability of this species to *in vitro* storage is needed.

In the next first section of the results there is a photo gallery of the 16 treatments (see for number of treatments table 1) after 2 months of slow growth condition (upper part) and at the end of the following regeneration period (lower part). The same treatment at 8 °C (on the left) and 22 °C (on the right). For example in the first set of photos treatment 1 and 9, correspond to L30-8H and L30-22H, respectively).

After two months of storage: treatment 1 and 9

Treatment 1 and 9: comparison in the regeneration phase

After two months of storage: treatment 2 and 10

Treatment 2 and 10: comparison in the regeneration phase

After two months of storage: treatment 3 and 11

Treatment 3 and 11: comparison in the regeneration phase

After two months of storage: treatment 4 and 12

Treatment 4 and 12: comparison in the regeneration phase

After two months of storage: treatment 5 and 13

Treatment 5 and 13: comparison in the regeneration phase

After two months of storage: treatment 6 and 14

Treatment 6 and 14: comparison in the regeneration phase

After two months storage: treatment 7 and 15

Treatment 7 and 15: comparison in the regeneration phase

After two months storage: treatment 8 and 16

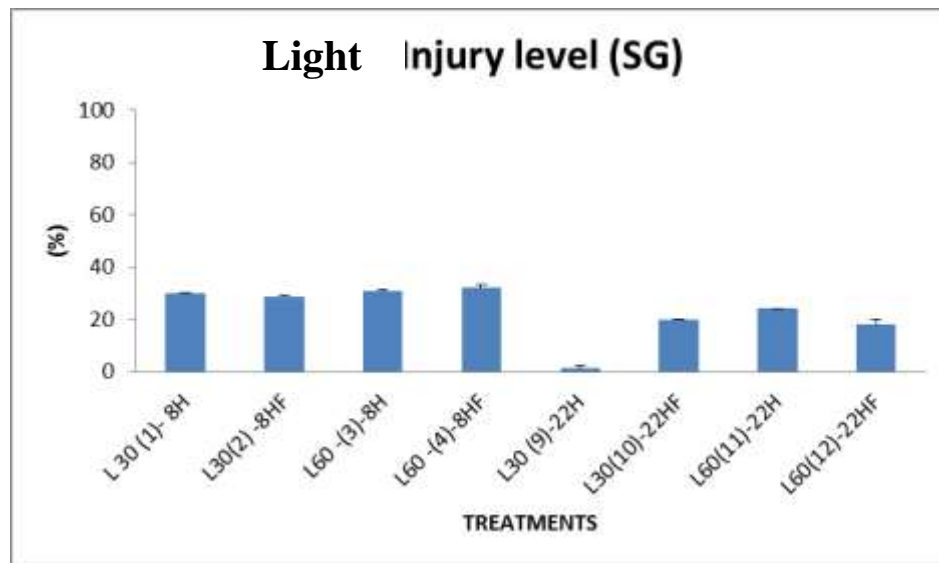**Treatment 8 and 16: comparison in the regeneration phase**
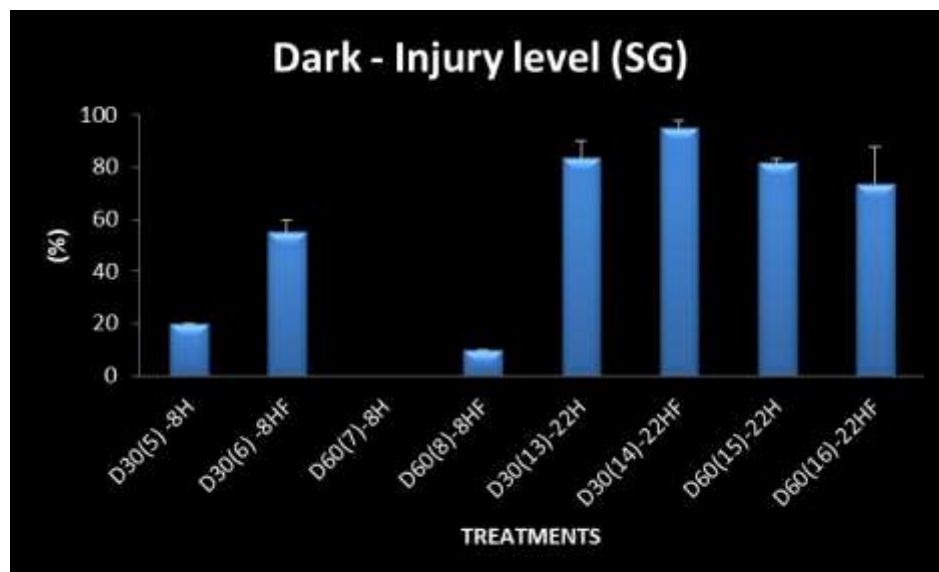
Fig 1

Fig 2

In figures 1 and 2 are reported the injury level percentages, induced by treatments under the slow growth conditions in *P. alba* (clone 6k3) explants. Treatment 9, having sucrose 30 g/L in culture medium with BAP at 22°C, recorded the lowest value for injury, in comparison to the rest of the treatments under light conditions. Regarding the level of injury in dark conditions, treatment 7, having sucrose 60g/L in culture medium with BAP at 8°C, recorded (0%) of injury level in comparison to rest of the treatments. The explants in treatment 9 appear lightish green in color with no signs of injuries.
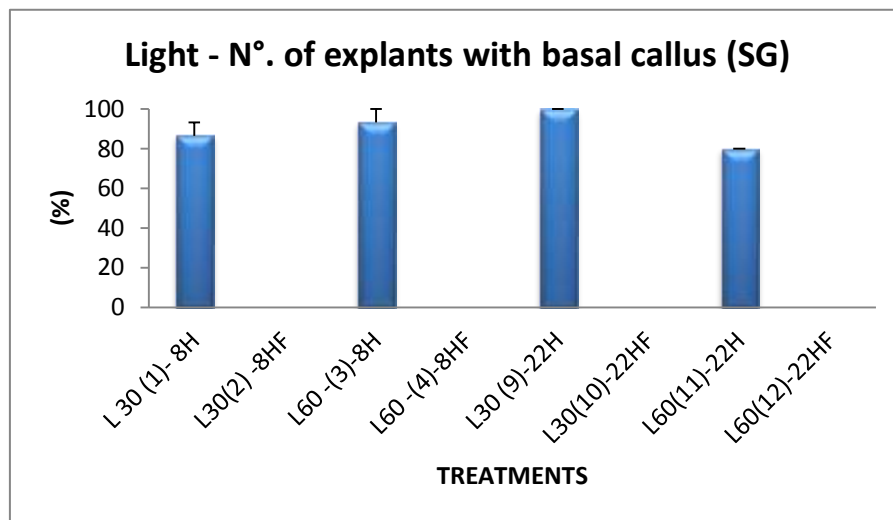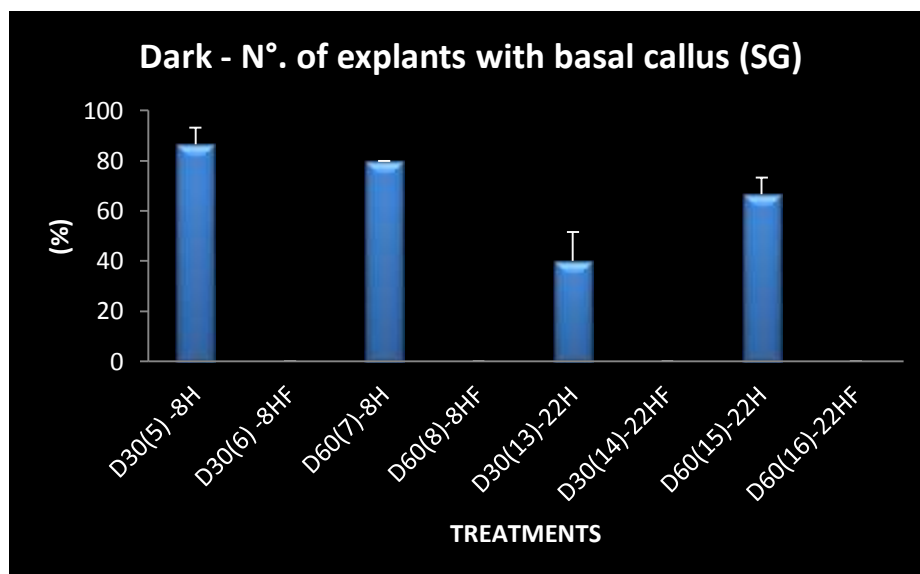
Fig 3

Fig 4

In figures 3 and 4 are reported the percentages of explants forming basal callus. It can be inferred that addition of BAP in culture the medium stimulates the formation of the basal callus. Treatments lacking BAP showed no callus formation at all. The size of the basal callus was different under the light/ dark and at 8°C/ 22°C. Explants under the Light conditions and at 22°C formed larger basal callus than at 8°C. On the other hand, treatments under dark conditions at 8°C/ 22°C formed basal callus but their size was smaller as compared to the treatments under light.

Fig 5

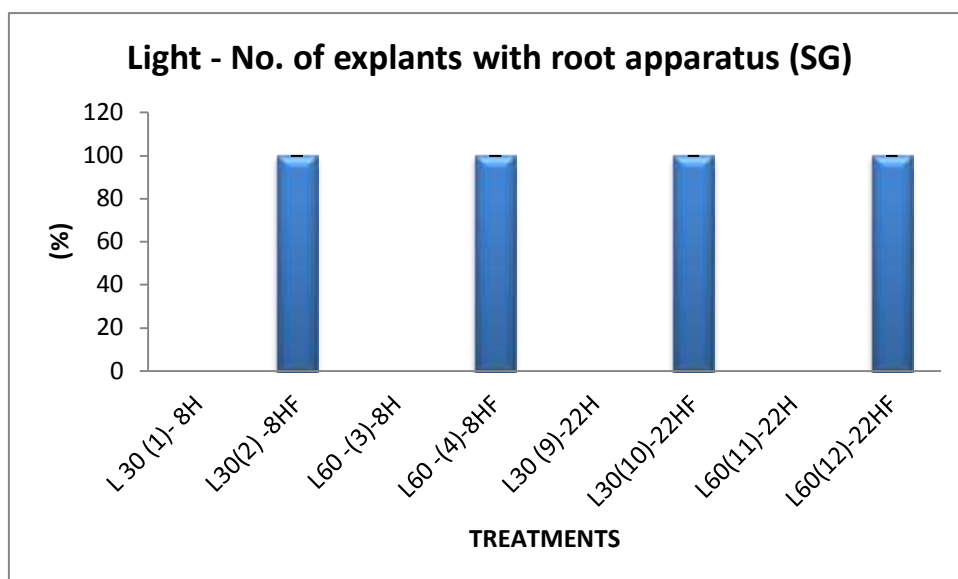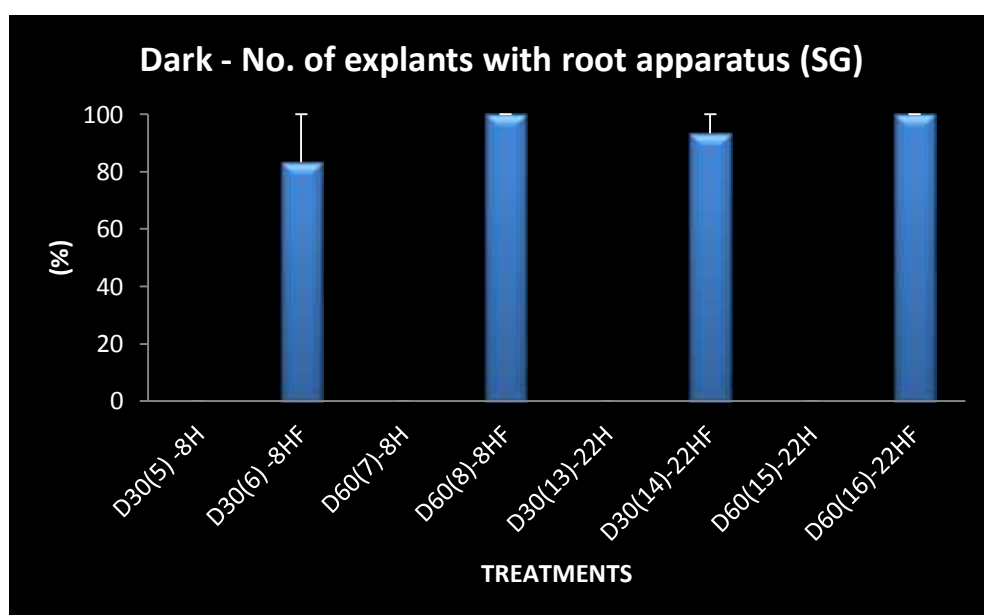**Fig 6**

In figures 5 and 6 are reported the data about the number of explants that formed root apparatus during the two months slow growth storage period. We can see that the treatment without BAP stimulates the formation of the root apparatus in most of the explants. On the contrary, treatments with BAP failed to form any roots at all. At 22°C, the treatments without BAP formed very robust root apparatus in comparison with treatments at 8°C and without BAP under light conditions. The treatments at 8°C/ 22°C under the dark conditions formed weak root apparatus in comparison with rest of the treatments.

Fig 7
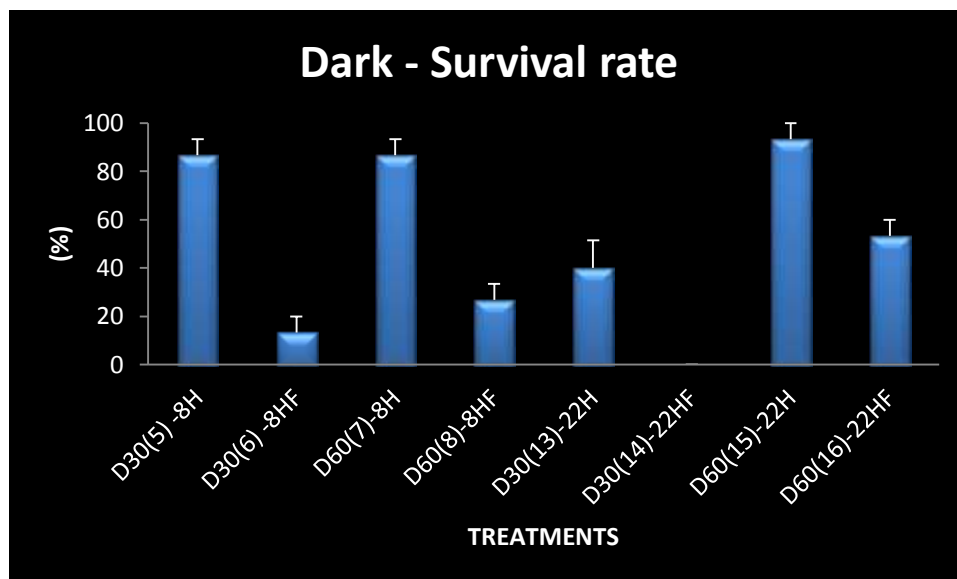
Fig 8

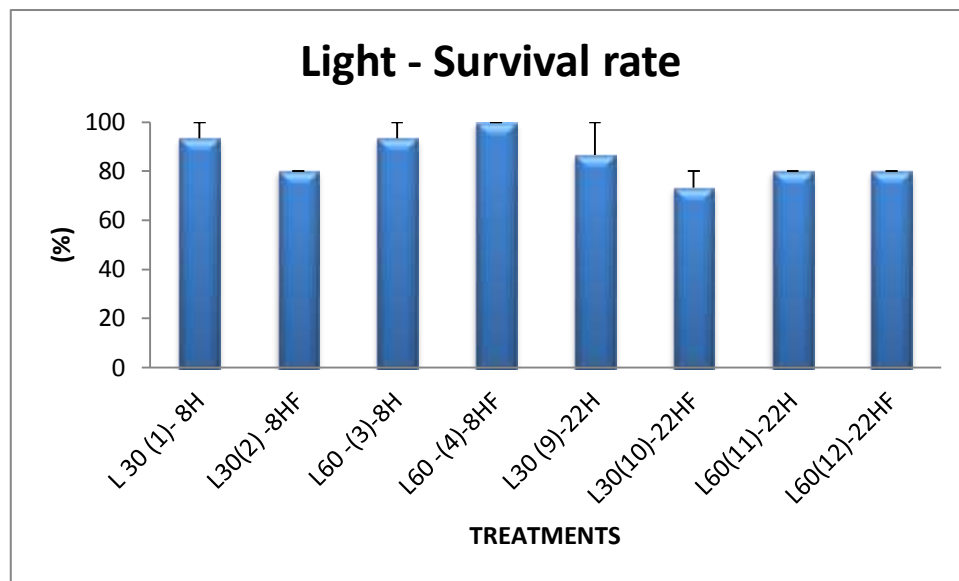In figures 7 and 8 are reported the survival (%) of *Populus alba* (clone 6k3) after the three subcultures in regeneration phase, after storage under different slow growth conditions. The treatments under the light conditions generally show very good survival values regardless of the presence of BAP, temperature and sucrose concentrations and the values were more homogeneous as compared to the treatments under the dark. The highest rate of survival (90%) was recorded for treatment 4, with sucrose 60 g/L in culture medium, lacking BAP at 8°C under the light conditions. The highest percentage of survival was recorded for treatment 15 , having sucrose 60 g/L in culture medium with BAP, whereas the treatment 14, having sucrose 30 g/L in culture medium and lacking BAP at 22°C recorded (0%) of survival under the dark conditions which was the lowest rate of survival among all other treatments.
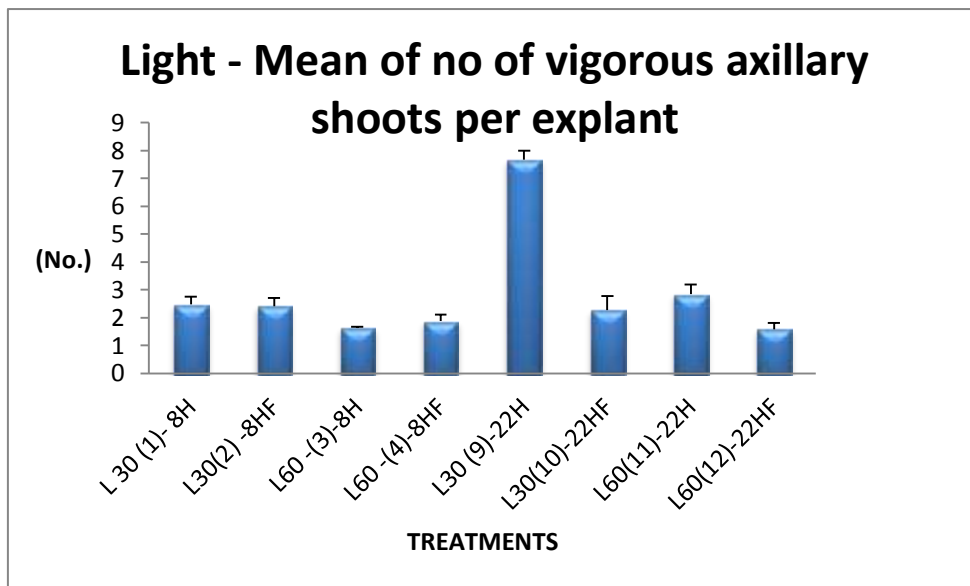
Fig 9
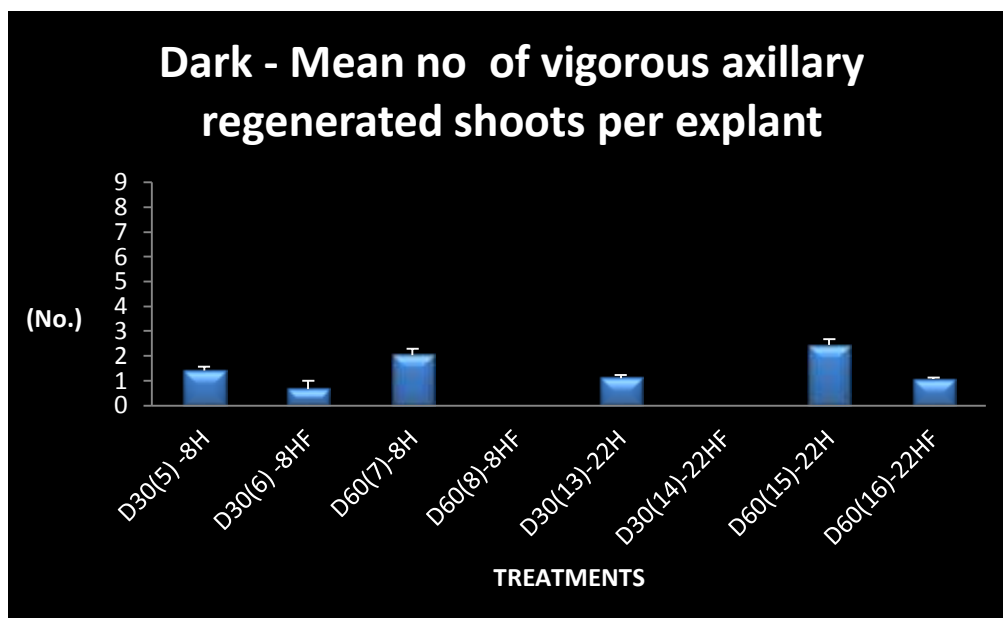
Fig 10

In figures 9 and 10 are reported the data regarding vigorous regenerated shoots per each explant at the end three subcultures for *P. alba* (clone 6k3). Treatment 9, having sucrose 30g/L, in culture medium with BAP, recorded highest mean no of axillary shoots (7.66) in respect to other treatments at 22 ℃ and light conditions. On the other hand in treatment treatment 15, having sucrose 60 g/L in culture medium with BAP at 22°C   was recorded the highest mean no of axillary shoots with respect to rest of treatments under the dark conditions.
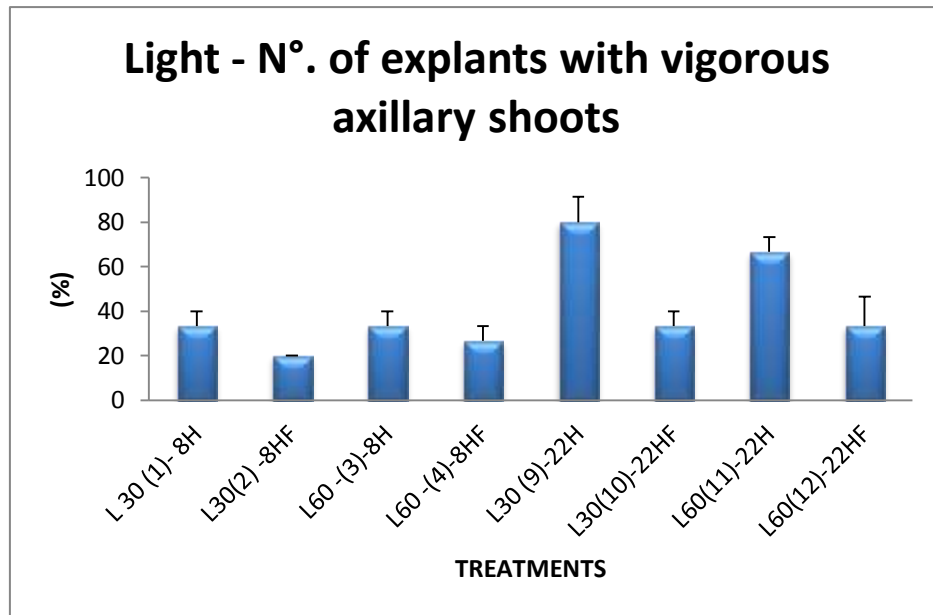
Fig 11
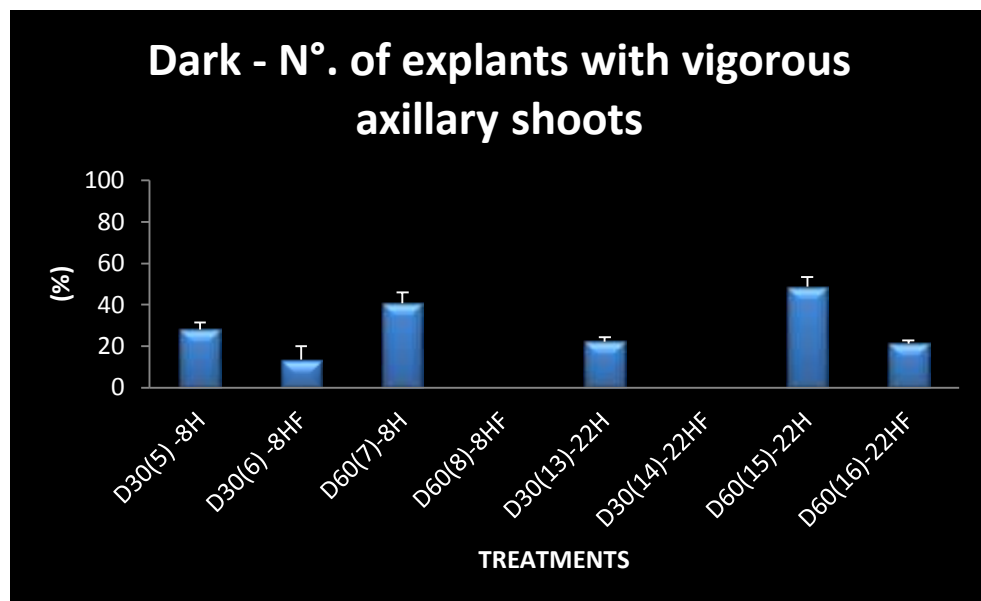
Fig 12

In figures 11 and 12 are reported graphically results regarding the explants with vigorous axillary shoots in *P. alba* (clone 6k3). Treatment 9, having sucrose 30 g/L in culture medium with BAP at 22°C gave the highest value (83%) of explants with vigorous axillary shoots, under the light conditions. The best treatment in dark conditions was treatment 15, having sucrose 60 g/L, in culture medium with BAP at 22°C recording (40%) of explants with vigorous axillary shoots.

Table 1. Results of statistical analysis on all the treatments: McKinney Index (%) at the end of two month slow growth period

Treatments	Means	
L30(1)-8H	30	AB
L30(2)-8HF	28.66	AB
L60(3)-8H	30.66	AB
L60(4)-8HF	32	AB
L30(9)-22H	1.33	A
L30(10)-22HF	20	A
L60(11)-22H	24	AB
L60(12)-22HF	18	A
D30(5)-8H	20	A
D30(6)-8HF	55	BC
D60(7)-8H	0	A
D60(8)-8HF	10	A
D30(13)-22H	83.33	CD
D30(14)-22HF	95	D
D60(15)-22H	81.66	CD
D60(16)-22HF	73.33	CD

*Values sharing a letter in the group label are not significantly different at the 5% Level.

30 and 60 = sucrose concentrations (g/L); H and HF = presence or absence of hormone (0.66 mg/L); L and D= Light and Dark conditions

From the table 1, it can observe that treatment 13-14 gave the highest level of McKinney index (the highest injury) in respect to the rest of the treatments. All these treatments induced significantly higher necrosis in comparison with other treatments under at 8 ℃ and 22 ℃ under the light conditions. The dark and room temperature seem to have a synergistic effect in negative way in these conditions.

Table 2. Results of statistical analysis on all the treatments: survival (%) in the regeneration phase after three subcultures after the slow growth

Treatments	Means	
L30(1)-8H	93.33	A
L30(2)-8HF	80	AB
L60(3)-8H	93.33	A
L60(4)-8HF	100	A
L30(9)-22H	86.66	AB
L30(10)-22HF	73.33	ABC
L60(11)-22H	80	AB
L60(12)-22HF	80	AB
D30(5)-8H	86.66	AB
D30(6)-8HF	13.33	CD
D60(7)-8H	86.66	AB
D60(8)-8HF	26.66	CDE
D30(13)-22H	40	BCDE
D30(14)-22HF	0	C
D60(15)-22H	93.33	A
D60(16)-22HF	53.33	ABCDE

*Values sharing a letter in the group label are not significantly different at the 5% Level.

30 and 60 = sucrose concentrations (g/L); H and HF = presence or absence of hormone (0.66 mg/L); L and D= Light and Dark conditions

From the table 2, it can be inferred that the highest percentage of survival of explants was recorded in the treatment 4, sucrose 60 g/L in medium lacking BAP under the light conditions. The lowest rate of survival (0%) was observed in the treatment 14, sucrose 30 g/L in culture medium lacking BAP under the dark conditions. Regarding the significance of the different treatments, treatment 14 was highly significant with respect to most of other treatments.

Table 3. Explants with vigorous axillary shoots (%) in the regeneration phase after three subcultures

Treatments	Means	
L30(1)-8H	33.33	ABC
L30(2)-8HF	20	AB
L60(3)-8H	33.33	ABC
L60(4)-8HF	26.66	AB
L30(9)-22H	80	C
L30(10)-22HF	33.33	ABC
L60(11)-22H	66.66	BC
L60(12)-22HF	33.33	ABC
D30(5)-8H	28	AB
D30(6)-8HF	13.33	A
D60(7)-8H	40.66	ABC
D60(8)-8HF	0	A
D30(13)-22H	22.2	AB
D30(14)-22HF	0	A
D60(15)-22H	48.66	ABC
D60(16)-22HF	21.33	AB

*Values sharing a letter in the group label are not significantly different at the 5% Level.

30 and 60 = sucrose concentrations (g/L); H and HF = presence or absence of hormone (0.66 mg/L); L and   D= Light and Dark conditions

From the table 3, regarding the treatment with highest values in terms of vigorous axillary shoots, treatments 1, 3, 7, 9, 10, 11, 12 and 15 were the best treatments.

Table 4. Results of statistical analysis on all the treatments: explants with Root Formation (%) at the end of two months the slow growth period

Treatments	Average	
L30(1)-8H	0	A
L30(2)-8HF	100	B
L60(3)-8H	0	A
L60(4)-8HF	100	B
L30(9)-22H	0	A
L30(10)-22HF	100	B
L60(11)-22H	0	A
L60(12)-22HF	100	B
D30(5)-8H	0	A
D30(6)-8HF	83.33	B
D60(7)-8H	0	A
D60(8)-8HF	100	B
D30(13)-22H	0	A
D30(14)-22HF	93.33	B
D60(15)-22H	0	A
D60(16)-22HF	100	B

*Values sharing a letter in the group label are not significantly different at the 5% Level.

30 and 60 = sucrose concentrations (g/L); H and HF = presence or absence of hormone (0.66 mg/L); L and D= Light and Dark conditions

From the table 4, in terms root formation percentage, we can see that all those treatments in which the culture medium lacks BAP, the explants formed the root apparatus. It was also noted that the vigor of the root apparatus varied greatly in terms of size under light/dark and 8 °C and 22 °C. In the treatments under the light conditions and 22 °C in absence of BAP, the explants form very healthy root system.

Table 5. Results of statistical analysis on all the treatments: explants with basal Callus Formation (%) at the end of two months slow growth period

Treatments	Means	
L30(1)-8H	86.66	B
L30(2)-8HF	0	A
L60(3)-8H	93.33	B
L60(4)-8HF	0	A
L30(9)-22H	100	B
L30(10)-22HF	0	A
L60(11)-22H	80	B
L60(12)-22HF	0	A
D30(5)-8H	86.66	B
D30(6)-8HF	0	A
D60(7)-8H	80	B
D60(8)-8HF	0	A
D30(13)-22H	40	C
D30(14)-22HF	0	A
D60(15)-22H	66	BC
D60(16)-22HF	0	A

Values sharing a letter in the group label are not significantly different at the 5% Level.

30 and 60 = sucrose concentrations (g/L); H and HF = presence or absence of hormone (0.66 mg/L); L and D= Light and Dark conditions

From the table 4, we can see that all those treatments in which the medium was supplemented with BAP, explants formed the basal callus. It was noteworthy to mention that the size of basal callus varied greatly under light/dark and 8 ℃ and 22 ℃ conditions, respectively. Treatments under light conditions and at 22℃ in culture medium with BAP, regardless the sucrose concentrations, formed very big basal callus.

Table 6. Results of statistical analysis on all the treatments: mean no of vigorous shoots per explant in the regeneration phase after three subcultures

Treatments	Means	
L30(1)-8H	2.46	AB
L30(2)-8HF	2.41	AB
L60(3)-8H	1.63	ABC
L60(4)-8HF	1.86	ABC
L30(9)-22H	7.66	D
L30(10)-22HF	2.26	AB
L60(11)-22H	2.83	B
L60(12)-22HF	1.58	ABC
D30(5)-8H	1.4	ABC
D30(6)-8HF	0.66	AC
D60(7)-8H	2.03	AB
D60(8)-8HF	0	C
D30(13)-22H	1.11	ABC
D30(14)-22HF	0	C
D60(15)-22H	2.43	AB
D60(16)-22HF	1.06	ABC

Values sharing a letter in the group label are not significantly different at the 5% Level.

30 and 60 = sucrose concentrations (g/L); H and HF = presence or absence of hormone (0.66 mg/L); L and D= Light and Dark conditions

From the table 6, treatment 9, having sucrose 30 g/L in culture medium with BAP under the light conditions and at 22 ºC produced the overall highest mean (7.66) no of vigorous axillary shoots per explant and shows high significance in respect to other treatments. These data indicate that BAP provided in the storage culture medium is highly useful to induce the following multiplication during subculturing.

6.3 Slow Growth Experiment with Walnut

To perform the slow growth experiment of walnut, seedling of cultivar Sorrento, *in vitro* growing shoots were used as starting material. The experiments were carried out only in dark conditions at 5 ℃ and 22 ℃.

Below in the following pages, the data regarding the McKinney Index and Survival are shown in the form of tables and its subsequent interpretation.

Table 1.McKinney Index (Injury level) at the end of two months slow growth.

Treatments	Means	
30-HF- 5℃	78.41	A
30-H-5℃	76.00	A
60-HF-5℃	61.67	A
60-H-5℃	60.00	A
30-HF-22 ℃	61.67	A
30-H-22 ℃	60.00	A
60-HF-22℃	52.67	A
60-H-22℃	50.71	A

Values sharing a letter in the group label are not significantly different at the 5% Level.
30 and 60 = sucrose concentrations (g/L); H and HF = presence or absence of hormone (0.4 mg/L);

From the table 1 it can be inferred, that in terms of injury level, almost all the treatments showed the same level of injury percentages. It means that there is no significant effect of sucrose concentration, temperature or presence of BAP on the slow growth of walnut for two months under the dark conditions in terms of injury levels.

Table 2. McKinney Index (Injury level) at the end of three months slow growth.

Treatments	Means	
30-HF- 5ºC	100.0	A
30-H-5ºC	100.0	A
60-HF-5ºC	65.0	C
60-H-5ºC	83.0	B
30-HF-22 ºC	100.0	A
30-H-22 ºC	100.0	A
60-HF-22ºC	84.5	B
60-H-22ºC	84.5	B

Values sharing a letter in the group label are not significantly different at the 5% Level.

30 and 60 = sucrose concentrations (g/L); H and HF = presence or absence of hormone (0.4 mg/L);

From the table 2, it can be observed that the best treatment, giving the lowest injury percentage (65%) was 60 g/L of sucrose without BAP and at 5 ºC. The worst treatments, giving the 100% of injury, were the treatments with 30g/L, regardless the combination with the other treatments.

Table 3. Survival (%) at the end of two months slow growth

Treatments	Means	
30-HF- 5ºC	57.78	C
30-H-5ºC	65.56	BC
60-HF-5ºC	94.44	A
60-H-5ºC	100.0	A
30-HF-22 ºC	63.34	BC
30-H-22 ºC	87.50	AB
60-HF-22ºC	91.50	AB
60-H-22ºC	84.52	AB

Values sharing a letter in the group label are not significantly different at the 5% Level.

30 and 60 = sucrose concentrations (g/L); H and HF = presence or absence of hormone (0.4 mg/L);

From the table 3, it can be observed that, in general, 60g/L sucrose were more effective in allowing survive of the explants, with the exception of the treatment 30-HF-22 ºC.

Table 4. Survival (%) at the end of three months slow growth

Treatments	Average	
30-HF- 5ºC	0.0	D
30-H-5ºC	0.0	D
60-HF-5ºC	26.6	A
60-H-5ºC	6.6	CD
30-HF-22 ºC	0.0	D
30-H-22 ºC	0.0	D
60-HF-22ºC	10.0	BC
60-H-22ºC	15.0	BC

Values sharing a letter in the group label are not significantly different at the 5% Level.

30 and 60 = sucrose concentrations (g/L); H and HF = presence or absence of hormone (0.4 mg/L);

From the table 4, it can be inferred that only the treatments with 60g/L sucrose allowed surviving of the explants, regardless the other treatments applied and the highest survival was obtained with the treatment 60-HF-5ºC. Thus, the highest concentration of sucrose used (60g/L) seems to be the critical factor from which to start for further experiments to develop a protocol for slow growth conservation of the explants in walnut.

Fig. 1. Two months and three months slow growth storage comparison of *J.regia* **cv "Sorrento"** *in vitro* **explants**

2 months

3 months

6.4 Cryopreservation Experiment with Walnut

In the present research study, a cryopreservation experiment was also performed with *in vitro* shoot apices and nodal segments of walnut. Experiment was carried out using "Encapsulation Dehydration Technique"**.**

To perform the experiments, shoot tips and single nodal stems were first excised from the explants and cultured on the 10 ml medium containing Petri dishes under the dark for one day at 5°C. After that the solution of sodium alginate was prepared. The shoot apices and nodal segments were then put into the sodium alginate solution and leave it for some time. In another container a solution of the calcium chloride was prepared. In order to get beads, a micropipette (with cutting on the end) was used to take shoot apices or nodes from sodium alginate solution. When single drops containing shoot apices or nodes were drop from micropipette in to the calcium chloride solution, beads were seen to form instantly. The beads were leave for 30 minutes in the calcium chloride solution in order to ensure its proper formation.

Due to the availability of the walnut material, only a 0.75 molar solution of sucrose was tested in this preliminary cryopreservation experiments, because sucrose at this concentration was shown to be suitable in other woody species. For successful cryopreservation protocol, it is necessary to have water content of plant tissues between 18-20%. For this purpose, the beads were cultured for one and two days in 0.75 molar solution of sucrose. After one or two days of sucrose treatment, the beads were transferred to flow hood for air desiccation. The maximum time for the desiccation was fixed at 6 hours. In order to get the complete picture of water content in the beads, a calibration curve was also performed. For calibration curve the beads without explants were removed after every one hour from flow hood, weighted and then oven dry to draw the calibration curve. For calibration curve we use the Fresh weight and Dry weight**.**

Calibration Curves for water content

In cryopreservation the content of water in the explant before immersion in LN is critical and it should be around 20%. In order to define the time necessary to reduce the water content in encapsulated beads with explants, two calibration curves were generated depicting the water content with respect to time of the beads left for air drying under the laminar flow. The fig. 1 and 2 below shows these data.
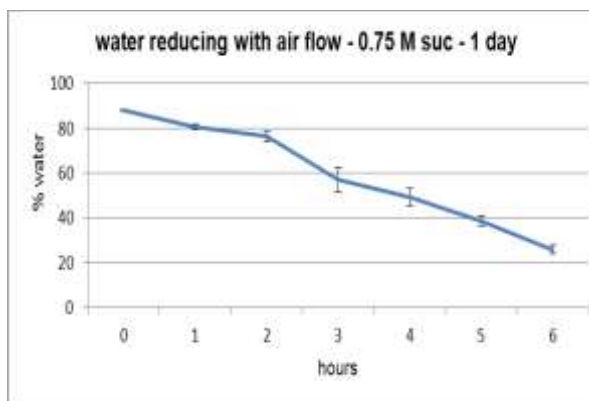

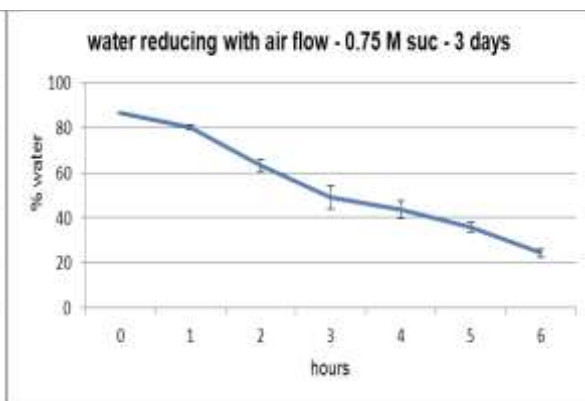**Fig. 1**   **Fig. 2**

6 hours of desiccation in the laminar flow allowed obtaining 20% of water content in the alginate beads. However, after the immersion in the liquid nitrogen, no shoot apices survived. Thus, further experiments are necessary to develop a suitable cryopreservation protocol for walnut shoot apices.

Overview of the Cryopreservation Experiment for *J.regia*
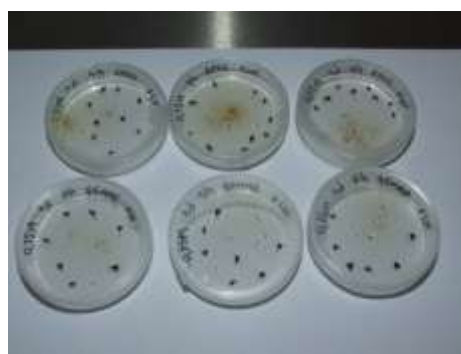
7.0 Conclusion

In this work, the possibility to store *in vitro* culturing shoots of *P. alba* (clone 6k3) and *J. regia*, cv. 'Sorrento' has been studied, whereby the effect of storage temperatures 8°C and 22°C, the influence of light and dark conditions, and the effect of plant growth regulators together with different concentrations of sucrose in the culture medium were assessed.

On the bases of results and discussion, we can conclude that generally the light conditions were found to be more suitable for slow growth storage of the *P.alba* (clone 6k3) for two months as compared to dark conditions. It can be also concluded that our experiments were not good in terms of long term conservation. Though in literature it was found that *P. alba* × *P. grandidentata* conservation could be prolonged up to 5 yr only when the culture medium was supplemented with a small amount of BA (Son et al. 1991). Similarly some scientists have successfully conserved other poplar species successfully up to six months irrespective of the light conditions. We know that in *in vitro* studies, sometime responses of explant material to certain conditions are highly genotype dependent. Sometimes even two genotypes of the same species behave very differently under the same set of *in vitro* conditions. As far as *P.alba* (clone 6k3) was concerned, we found no reported study on its *in vitro* conservation till this date. May be the *P.alba* (clone 6k3) is showing high negative genotypic response in terms of slow growth conservations as compared to other *P.alba* clones studied before. The other reason could be the accumulation of the ethylene in the storage containers. We use the standard glass containers for the storage of the explant materials, in literature there are reported studies on slow growth storage of *in vitro* plant material in special containers which can somehow allow the movement of ethylene from the storage containers to outside environment, ultimately reducing the negative effect of ethylene on the stored material. The effect of ethylene was most obvious under the dark conditions in treatments supplemented with BAP, although the explants appear green, but the leaves were found to bend inwards and during the transferring to regeneration medium, all the leaves falls down, the most obvious sign of ethylene accumulation in glass jars. In our experiment we choose only one temperature in terms of slow growth i.e. 8°C to see whether it could have impact on the plant metabolic activities. Generally it is reported, that at temperature below 10°C, most of the plant metabolic activities ceases to function, may be in our case the 8°C temperature fails to stop all the metabolic activities of *P.alba* (clone 6k3), and as a result the explants show

highly negative responses in terms of McKinney index at 8°C especially under the light conditions. The highly positive response in terms of mean number of axillary shoots per each explants in treatment 9, having 30 g/L of sucrose together with BAP in medium at 22°C, could be due to the fact that BAP could be uptake by explants in optimal manner at higher temperatures as compared to 8°C.Generally in most of the treatments we found the axillary shoots sprouting at the same rate (not very good), except those treatments in which we lost all the material. The most obvious reason for this could be the due to the fact that, we use *in vitro* rooted material for our experiments, and we know that root tips are involved in the biosynthesis of cytokinines. In other terms, we use the explants which already have endogenous cytokinines. For future experiments with clone 6k3, the 4 °C and multiplication clumps under dark conditions, could be the good way in terms of slow growth conservation for this clone. Because we observed (data not presented) that after three months of storage of the *P. alba* (clone 6k3) the clumps appear green and healthy. Therefore the use of clumps for this clone in slow growth conservation needs further investigation.

In terms of walnut, we found out that the rate of survival greatly varies with time of explants exposed to slow growth conditions. The explants stored for two months gives the highest percentages of survival as compared to the explants in three months storage.

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