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Essay of in vitro propagation of a medicinal plant *Pistacia vera* L.

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ABSTRACT

Pistachio nut (Pistacia vera L.) is a woody species of arid and semi-arid areas, has commercial interest, including product mastic resins and essential oils used in pharmacology. In Algeria, despite many pistachio-purpose zones, this species has been abandoned for various reasons including problems encountered during its propagation. The purpose of this study the key steps to obtaining vitro plants of P. vera L. by a vitro germination and axillary budding. Two parameters are tested: material plant (seeds and buds) and the composition of culture media. Immature seeds of P. vera L were disinfected after suffering a mechanical scarification and cultured on three different media. The introduction of axillary buds and their development were observed in all culture media tested at variable rates. Contaminated tubes were discarded and were the subject of a study to identify the microbiological contaminant responsible is a fungus (Penicillium).

Keywords: Pistacia vera L., Micro propagation, Germination in vitro, axillary budding, Penicillium.

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INTRODUCTION

Pistachio fruit is a well known Mediterranean species, naturalized and cultivated in our latitude since ancient times [10]; it is interesting because of its physiological capabilities that offer the ability to develop the arid and semiarid Algerian.

The widespread cultivation of the pistachio tree, its improvement and success depend upstream: the knowledge of plant material (figure 01) and the development of reliable techniques of multiplication and production of plants and downstream of the pipe and the maintenance of orchards (figure 02) [4].

Figure1: Cluster of pistachios in the region Ouled Mimoun (Tlemcen)
Figure2: Pistachio nut tree an orchard in of Ouled Mimoun (Tlemcen, Algeria).

Propagating trees by the vitro culture is not widespread in the world compared to other fruit species [1], as it faces several problems related to the choice of explants, the initiation aseptic necrosis of apical buds and especially the problems of contamination.
Also rooting explants remains a critical step in vitro culture, because its quality determines the future viability of the plant.

The objective of our ambition, consists to production of plans by in vitro culture (vitro germination and auxiliary budding), and the study of certain parameters (source, medium and culture conditions).

MATERIAL AND METHOD

Plant material

Buds were collected from subject's juveniles (seedlings 2 years) of seeds of *Pistacia vera* from el Fhoul (Tlemcen) and immature seeds from Sfisef (Sidi Bel Abbes) (figure 03, 04). Portions of the stem with buds are stripped of their leaves with a scalpel, then Fragmented in knots about 1 cm long, containing at least one bud.

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Figure 03: Sowing of two years (El Fhoul) Figure 04: Seedling 1 year (of Sidi Bel Abbes)

Disinfection of plant material

Exogenous and endogenous infections have always limited the in vitro introduction of explants in woody species. These two sterilization techniques were tested on our plant material. The first is the basis of sodium hypochlorite (Na ClO), the second involving a very powerful disinfectant, mercuric chloride ($HgCl_2$) (Table 01).

Table 01: Sterilization techniques of plant

Plant organ	Disinfection methods	Pre disinfection	Disinfection	Durations of disinfection	Post disinfection
Segments	Method 1	-	NaClO (2%) + tween 20	15 mn	3 rinses with distilled water
	Method 2	Pure Ethanol 2 mn	NaClO (20%)	10 mn	5 rinses
Nodes	Method 1	-	-$Hgcl_2$(2,5 g/l) -$Cacl_2$(2,5 g/l)	20 mn 02 mn	3 rinses

Culture media

The intake of minerals, vitamins, amino acids and phytohormones culture media tested is based on the culture stage. Its role is to ensure the nutrition of explants. For this experiment we have used the mineral compositions given in Table 02.

Table 02: Composition of culture media

Solutions Macrominerales	Solutions Microminerales	vitamins Solutions	Growth regulators	organic components	Browning inhibitors
MS MS/2 MS/4 QL DKW	MS DKW	MS DKW GBG	Auxines (AIB, ANA). Cytokinines (BA, BAP, KIN). Gibbérellines (GA_3)	Saccharose Agar-agar Gelrite	$AgNO_3$ Acide ascorbique

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The pH of the culture medium is adjusted using sodium hydroxide (NaOH) or 5.7 to 5.6 before the addition of agar agar. The tubes are sterilized in a dry oven at 150 ° C for 3 h. The prepared culture media are distributed in these tubes and autoclaved at 120 °C, under a pressure of 1.3 bar for twenty minutes.

Conduct of cultures and observation

The tubes have been used in the growth chamber with daily monitoring to eliminate those that are contaminated.

Identification of the contaminant

The importance of contamination observed on all tubes removed, forced us to identify the contaminant and responsible in order to know its nature, we conducted laboratory tests of identification made by the method of single spore based on four different backgrounds by their elemental composition and are: CDA (Czapek Dextrose Agar), **MEA** (Malt Extract Agar), **CYA** (Czapek Yeast Agar) and **G25N** (25% Glycérol Nitrate Agar).

RESULTS AND DISCUSSION

Influence of culture media on immature seeds

The best germination rate obtained was about 50% of hypocotyls with a height of 2 to 3cm (figure 05) and whatever the concentration of gibberellic acid (GA3) used in mineral medium Abous 3 where macronutrients diluted four times with added vitamins and microelements of muraschige and skoog (MS).

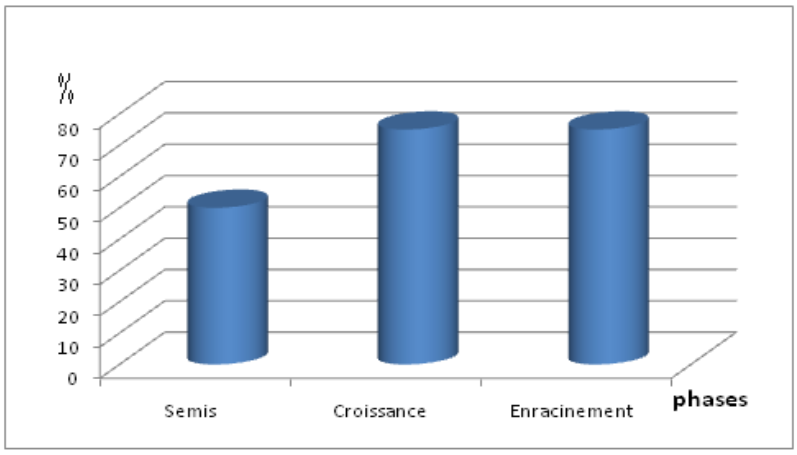

Figure 05: Effect of culture media on growth of immature seeds of *Pistacia vera* L

The MS medium/4 without AG3 or the presence of 2 mg/l displays the same germination percentage of about 16.66%, but with the addition of 10 and 20 mg/l of gibberellic acid low

viability rate not exceeding the 8.33% is recorded. On the other hand, the results obtained by Djedid [6] in this same medium supplemented with 10 mg/l of GA3 on seed *Pistacia atlantica* Desf.; displays a germination rate of around 72% and a rate of 68 % with 2 mg/l of GA3. According Abousalim and *al.*, [1], the MS medium/4 added to 2 mg/l of AG3 and MS/2 with 30mg/l of GA3 have achieved 100% germination.

The middle two About macro-element consisting of MS [13] diluted four times, led to good growth and good appearance of hypocotyls (figure 06), with a rate of about 75% and a length not exceeding 3 cm. Moreover, Kafkas and *al*. [8] showed that seeds treated with GA3 offer a larger diameter rod.

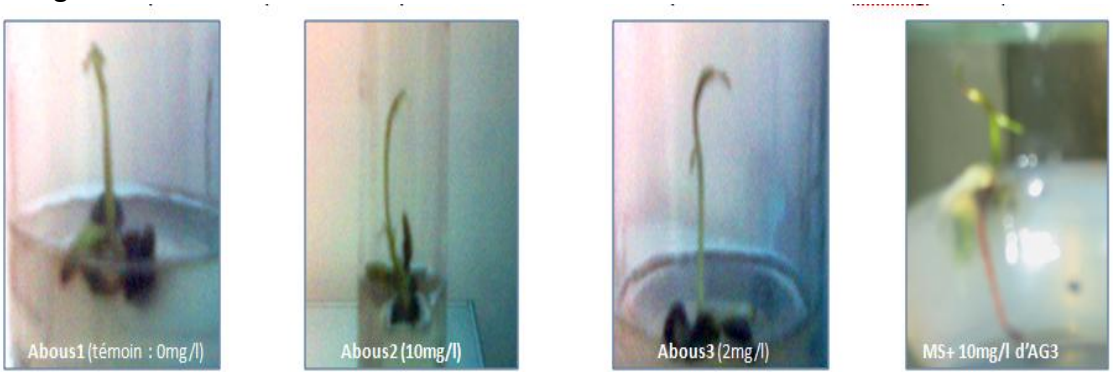

Figure 06: Reaction of immature seeds of *Pistacia vera* L

However, Abousalim and *al.*, [2] show that root growth appears to be stimulated by the presence of a medium rich in minerals and container in addition to gibberellic acid. The influence of light on root growth can only be indirect through the aerial part [9].

Influence of culture media

Responsiveness of the explants cultured in vitro, would depend on the species, source of explants, the composition of culture medium (figure 07), and the time of removal as well as the disinfection technique used [15, 7].

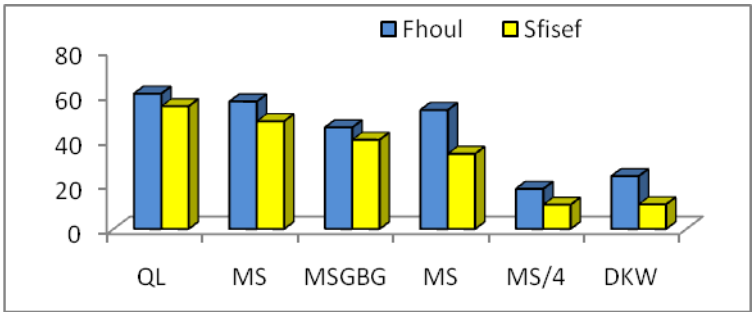

Figure 07: Effect of culture media on growth of micropropagated *Pistacia vera L* in three phases

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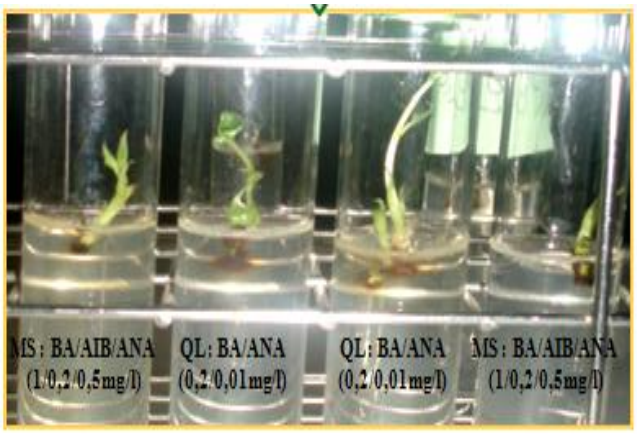

Figure 08: Response of buds on Initiation phase

The experiments in this part of the buds of the species *Pistacia vera* taken from young shoots can multiply within in a very short time a juvenile plants stage. Indeed, the juvenility of explants plays a key role in the success of this technique [3]. After 30 days, comments made and have shown that the middle of Quoirin and Lepoivre (QL) disinfected by the second method is based on sodium hypochlorite (Clo Na) seems more effective for a quick response bud rosette leaf (Figure 08). In fact 32.14% of the buds are reactive after 20 days of culture. Providing a solution of auxin-cytokinin in this same medium (with respectively 0.01 mg/l NAA and 0.2 mg/l BA) has been essential in organogenesis. The use of silver nitrate ($AgNO_3$) did not affect the normal development of the explants *P. vera* but it did not assume its role as to Browning because it indicates the appearance of 14.28%.

The multiplication of explants in MS medium added to the solution of vitamin Gomborg significantly improves bud growth with a rate of 53.33% but the high concentration of cytokinin (BA = 2.5 mg/l) and Gibberellic acid (GA3 = 2 mg/l) promotes the proliferation of auxiliary meristems and allows the production of clusters of buds with a length ranging from 1 to 4 cm (figure 09), in the heart of this phase the addition of GA3, which has dominant purpose of obtaining bouturable shoots (greater than 2 cm) is necessary for good root development.

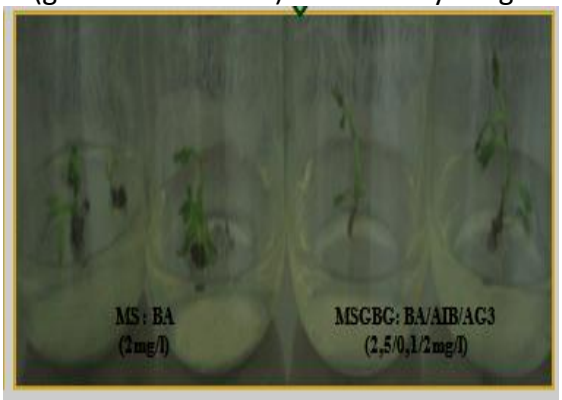

Figure 09: Proliferation of auxiliary meristems on Multiplication phase

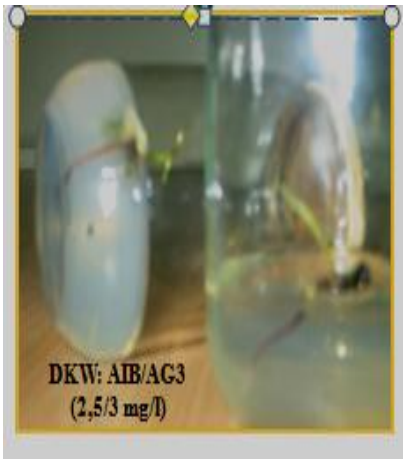

Figure 10: Rooting phase

For rooting of seedlings, the results obtained on DKW medium (figure 10) associated with 2.5 m/s from the AIB provides a very low percentage of rooting in the range of 23.66% compared with the auxin/cytokinin (NAA: 0.5mg/l, KIN: 0.5mg/l) which favors a rooting rate of 17.98%. And David [5] argued that auxin is the main factor involved in root formation in fruit and forest trees grown in vitro. Note that the auxin IBA is most appropriate when it comes to getting an intense root growth of *Pistacia vera* [11].

Identification of the contaminant

Disinfection method based of sodium hypochlorite (12%) provides a variable rate according to the reactivity culture medium and promotes a contamination rate of the order of 10.71% in initiation phase (figure 11). Compared to this method by a strong disinfectant mercuric chloride (HgCl2), even if does not cause contamination, allows a medium reactivity buds.

Figure 11: effects of media culture on the health status of explants Pistacia vera during the initiation phase

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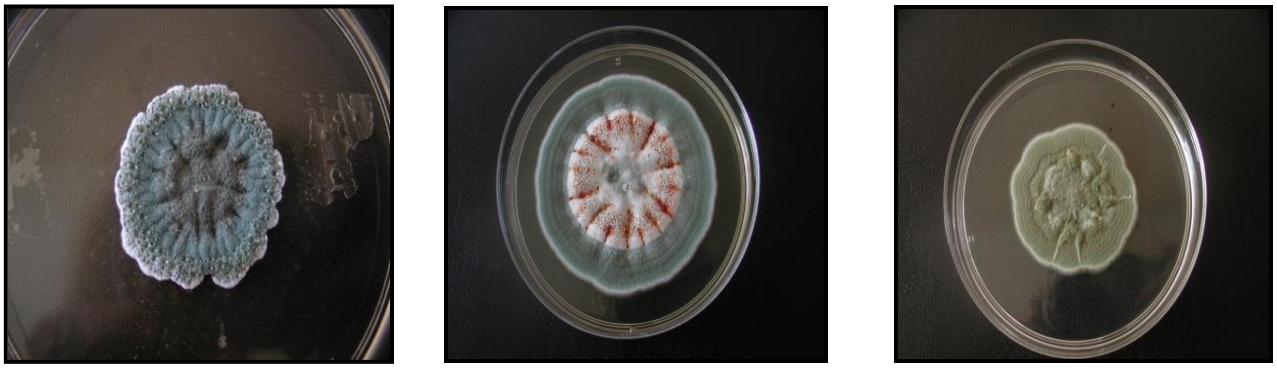

Figure 12: Identification of main *Penicillium* species infecting in vitro culture in the pistachio nut.

The identification of the contaminant responsible for the method of single spore enabled us to know their nature. This is actually a fungus that has a textured green, known as the genus *Penicillium.* We identified three *Penicillium* species namely: *Expansum*, *Verrucosum* and *Digitatum* (figure 12).

Table 03: The reaction of immature seeds after three stages of culture

	Factors / Phases	Macro	Micro	Vitamins	Phyto hormones mg/l	Other elements g/l	Terms of culture	Reaction (%)
G I V	Sowing	MS/4	MS	MS	AG_3 = 2	Sac : 30 Agar : 7	pH : 5.7 Photop: 16 h Temp: 25°c Lum : 2000 luxux	50
	Growth	MS/4	MS	MS	-	Sac : 30 Agar : 8		75
	Rooting	MS	MS	MS	AG3 = 10	Sac : 30 Agar : 8		75 et une longueur de +3 cm

Table 04: Results of the reaction of micro-cuttings after one, two and three months of culture

Phases	Middle variation	Hormonal Combination	Hormone Concentration (mg/l)	Reactions (%)	
				Fhoul	Sfisef
Initiation	QL	BA/ANA	0,2 / 0,01	60,69	55
	MS	BA/AIB/ANA	1/0,2/0,05	57,14	48,30
Multiplication	MS	BA	2	45,5	40
	MS +V(GBG)	BA/AIB/AG3	2,5/0,1/2	53,33	33,67
Rooting	MS/4	ANA/KIN	0,5/0,5	17,98	10,84
	DKW	AIB/AG3	2,5/3	23,66	11

The main causes of this infection are likely due to:

1 - The air quality often responsible for microorganism;
2- The nature and position of plant tissues which are covered on the surface of microorganisms, especially those near the ground (branches, roots ...)
3 - And the human body that carries multiple micro-organisms (skin, breathing ...).

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CONCLUSION

With the aim of achieving sufficient production and healthy, is more than necessary to focus on improving our true pistachio orchards through conventional methods (cuttings, grafting ...) and vitro methods (multiplication by auxiliary budding and embryogenesis and germination in vitro), which would no doubt be an asset in the hands of practitioners. Recent work undertaken in the laboratory allowed us to investigate the ability of pistachio multiplication by in vitro culture.

The main results obtained in this experiment can be summarized as follows:

- Good disinfection has been gained with the technique proposed by Yang and Ludders [16], based on the use of a solution of sodium hypochlorite (12%), which gives an appreciable reactivity explants but *Pistacia vera* generates parallel contamination.
- The culture medium Quoirin LEPOIVRE [14] and added to 2 mg/l of $AgNO_3$ allows obtaining a better response rates of around 60.69% and a rate of browning of the order of 14, 28%. The use of $AgNO_3$ as an antioxidant has not completely eliminated this problem is due to the phenolic extracts.
- The best rates and multiplier which is the order of 53.33% is caused by the middle of MS over the solution of vitamin Gomborg supplemented with 2.5 mg/l BA, 0.1 mg/l AIB and 2 mg/l GA3. For the rooting phase, the results are not satisfactory because it displays a small percentage of about 23.66% on the middle of Driver and Kuniyuki added to 2.5 mg/l IBA and 3 mg/l of GA3. This allowed the emergence of a radical puny after 20days of culture.
- The maturity of the seed is important because the immaturity at harvest usually results in a low initial germination but also by an inability to conservation [12].

Obtaining vitro-rooted plants allows us to enter the phase of acclimatization. This phase is to adapt these explants to the external environment in greenhouses but without artificial controlled conditions of temperature, humidity and photoperiod step could not be addressed.

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