In-vitro Propagation of Date Palm (*Phoenix dactylifera* L.) by Adventive Buds*

Talal Amin

Faculty of Agriculture, Tishreen University
Lattakia, Syria

ABSTRACT

Propagation of date palm of some local varieties (Khistaoui and Zahidi) was performed in the laboratory by means of the advantive buds where the upper parts (non-meristematic) taken from the young leaves surrounding the top bud. After, about five months of the basic cultivation, small, white and firm tissue protuberance were noticed emerging from within the explant's tissue. The protuberance were cultivated in another nutritive media. Tissue clusters of firm construction, rapidly divided and of meristematic nature, were obtained. These clusters were propagated to get the required quantity of the propagation material. In a later stage, the tissue meristematic clusters were cultured, and coherent mass of developed buds was obtained. The healthy green growths were cultivated in a suitable nutritive media to be enhanced to grow and develop and form strong roots. Great quantity of vitro plants capable of living and ready for hardening, were obtained.

A parallel histological study of the explants was performed, including various stages of development. The microscopic observations showed the cell division followed by clear growth of the explant. At the end of this stage, scattered, fine clusters appeared near the epidermis, consisting of very fine and active cells.

Tissue nodules emerged from the cell clusters. Those nodules were independent and of different shapes and of dense cell clusters, which formed a cohesive unit, and displayed an internal tissue differentiation. The development of the nodules resulted in tissue structures which were not uniformed in their size and activity and function, and then gave very fine meristems , which consist of meristematic dome and permordium leaves in which the paranchyme that contains scattered vascular bundles at different degrees of development.

INTRODUCTION

Date palm is propagated now by two main methods, the first is known as propagation by the somatic embryogenesis and the other is known as propagation by forming auxiliary buds.

Propagation by Somatic Embryogenesis:

This method is characterized by giving in-vitro plants, in, a comparatively, shorter time (20 -26). months, as well as, high propagation ranges. The primary plant materials can be cultivated any time of the year. But the most important disadvantage of this method is passing the embryogenesis callus, and its propagation, which enhances the possibility of mutations and abnormalities occurrence during the growing stage of the In-vitro plants, and which may appear in the fields later on (AI-Wassel, 2000).

Propagation by Auxiliary Buds:

One of this method's advantages is getting In-vitro plants highly identical, in their vegetative characteristics, with the mother plant. The disadvantages of this method are technical and related to the long time required to obtain In-vitro plants, in addition to the, relatively, low averages of propagation.

This, in addition to the fact that cultivation of plant's primary parts can not be done unless in the dormancy stage. (Beiiuchesne, 1997, Rhiss, personal contacts, 1998, 1999), which limits the flexibility of applying it all the year round.

It is worth mentioning that the explants treated by this method undergo serious technical problems, such as, browning and early appearance of the roots and the transparency phenomenon (Ait -Chitt, com. No.18); (Ait -Chitt 1996 and Rhiss, 1998 & 1999, personal contacts).

Due to the disadvantages of the two date palm propagation methods, a new method has to be applied by using the advantive buds of date palm, which depends mainly on generating renewed buds on the surface of the treated In-vitro explants, which do not normally produce buds (Gaspar, 1988; Margara, 1984).

The aim of applying a new technique is to provide various methods for propagating date palm via tissue culture, and improve the presently applied procedures. Nasser (1996) and AI wassel (2000) confirmed the necessity of developing the present propagation methods, to avoid the disadvantages as much as possible.

The second aim, and the most important one, producing commercial quantities of date palm of the desired varieties in syria (Amin, 2000).

MATERIALS AND METHODS

Preparing the Plant Material for Cultivation:

Offshoots of two local varieties (Khistaoui and Zahidi) were taken from the mother trees in AI-Jalaa nursery, AI-Boukmal, Der EI-zour. Primary preparation of the offshoots was performed outside the laboratory, where the fibers and the dead plant remainders were removed, as well as, the basic part, which includes the roots and soil, in addition to the upper mature parts.

In the laboratory, the leaves were removed gradually from the bottom to the top (Acropete), till reaching the top of the offshoot. The top of the offshoot was sterilized in a solution of sodium hypoclorite (5.25%) diluted by sterilized water at 1:1 for 20 minutes. Under a ordinary magnifier, in a luminar, the young leaflets and part of the core's tissue were removed gradually till reaching the top bud.

In this research, parts of the young fine leaflets surrounding the top bud, which their length does not exceed 2 -3 mm, were used, after removing the meristematic basic parts (Photo No.1).

The explants were cultivated various times in one year, according to different phonological stages of the mother plant such as dormancy stage, flowering stage. ..etc. Cultivation was repeated for four successive years proceeded by two years of initiative work .

The Nutritive Media and Cultivation Conditions:

The used nutritive media was composed of Murashige & Skoog 1962, since it is, on one hand, the most popular in date palm propagation by tissue culture, and on the other hand, it exceeds all the other solutions

in releasing formation of the new buds (Margara, 1984). The following chemicals were added to this solution:

a- Amino acids: these acids were added at various concentrations: araginine, espargine, glycine, adinine and glotamine. Adding the amino acids to the nutritive media enhances, strongly, the formation of the organs, in many cases (Margara, 1984).

b- Vitamins: the following vitamins were added at different quantities: biotine, pyrodoxine, nicotenic acid and thiamine, while the enozytol was, always, added at lOO mg/1. The kinds and concentrations of the amino acids and the vitamins differed according to the physiological status and development stage of the cultivated explants .Some of this adding of the acids was done in accordance with(Poulain ~, 1979) for its importance in enhancing formation of the new buds.

c- Other organic materials: sucrose was added at 30 -40 gr / I, and active charcoal at 0.3-3 gram I. The importance of the charcoal in adsorption of the inhibitors and growth preventers (Emst,1984; Wang et Hong, 1976; Anagostakis, 1974), is well known, as well as, in increasing the living explants and formation of the date palm's organs, when used at 3 gr/1 concentration (Tisserat, 1979), while the agar was used at 6 gr/1 concentration.

d- Growth regulators: The used growth regulators differed in kinds and concentrations according to the experiment's stages and the physiological status and development stage of the cultivated explants. Of the used auxins we mention: 2.4 D, ANA, NOA, AlA, AIB. And of the used cytochynines we mention: 2, ip, K, BAP.

Before adding the agar and the active charcoal, the PH degree was fixed at 5.8. ± 0.1. Then the solution was poured either in tubes (2.5 x 20 cm) at 17 ml per tube, or in special jars at 50 ml per jar. Then the nutritive media was autoclaved at 120 °c for twenty minutes.

After cultivating the explants in the suitable nutritive media, they were incubated in a growth room, in complete darkness at the early stages, and in specific light conditions in the advanced stages. The cultivated explants were kept in thermal conditions ranged between 26°c during darkness, for 8 hours; and 28 °c during illumination for 16 hours.

Each experiment included 40 -50 explants .Remarks were taken by measuring, counting and describing at the end of the experiment and during transplanting and for a period differed according to the aim of the experiment and its duration.

The Histological Study:

The specimens selected for histological analysis were fixed in a mixture of initially equal valums of solution A (chromic acid 300cc + water 200 cc) and of solution B (Acetic acid 200cc + formol 100cc + water 200cc). The fixed samples were rinsed for 24 hours in running water, dehydrated in a series of baths from 50 to 100% ethanol and embedded in paraffin. Sections were cut at 10~m thickness with rotary microtome, deparffined and stained in hematoxylin, safranin and aniline blue solutions. Observations were made under a leitz microscope

RESULTS

Formation of the pro-meristematic tissue:

The primary explants of 2-3 mm length were cultivated in the basic solution which contains the following auxin 2.4, D -ANA -NOA at different, and comparatively high concentrations, and the cytokenines 2ip -K -BAP at different and comparatively low concentrations.

The study of the tissues showed that the parts of the young leaflets are of uniformed-shape tissue structure, consists of small and compressed cells of consistent structure, in addition to one or more layer of prolonged and compressed cells forming the epidermis(Photo No. I).

After one month, the cultivated explants began to respond, showing small swelling which grew slowly. At the end of this stage, the responded explants became 6 -20 mm long and 4 - 8 mm wide. The responded explants formed about 50% of the total quantity of the cultivated explants, according to the date of the basic cultivation and the variety.

On nearly 65% of the responded explants, protrusions of white tissue swellings were noticed emerging from different places of the explants' interior tissues. These swellings formed the pro meristematic tissue, which is distinguished by its hardness and strong integration and its cells' welding and sticking to the mother explant at different length ranged between 1 -4 mm. This stage lasted about 5 months (Photo No.2).

The anatomic study of the, responded explants showed clear change in the tissue nature. The primary explant was subject to active cell division, followed by cell prolongation, which led to increase in the size of the explant and produced heterogeneous structure of this tissue .

The structure should big cells not uniformed in their size and compactness. Also, there were scattered cell clusters near the epidermis. These cells were distinguished by their fineness and compactness, which was considered the reason of the protrusions' formation, and consequently, the formation of the pro-meristematic tissue (Photo No.2) .

As for the date of the primary cultivation, it was noticed that the best date to obtain the best response of the explants coincided with the dormancy stage and the before of flowering stage, while the minimum response of the explants coincided with the fruiting stage.

Formation of the Pro- Meristematic Tissue:

The responded explants were re-cultivated in a new nutritive media to which cytokinenes, such as HAP, 21P, K were added at different and, relatively, high concentrations. Also, auxines such as NOA, ANA, AlA were added at different and small quantities.

After 2-3 months, relatively, big-sized, developed, white-colored, firm and strongly welded together tissue nodes appeared starting from the pro. meristematic tissue (Photo No.3).

Longitudinal sections of meristematic nodules showed the presence of meristematic islates which constituted by small cells with large and off centre nuclei. The meristematic isolates emerge from large and strongly vacuolized parenchymatous cells. There meristematic zones could develop into meristemes (Photo No.3).

Upon proceeding with breeding the meistematic tissue in the same nutritive media, and at the end of the formation procedure, tissue nodes easily separated and growing in all the direction taking different forms and sizes, were obtained. This development may coincide with a limited growth of the meristems, part of which appeared on the surfaces of these masses, while the other part remained inside the same tissue. Thus, the tissue generating the buds could be formed. This was considered the basic material for propagation.

Propagation of the Meristematic Tissue:

The primary meristemic tissue was cultivated in the propagation media which contained hormonal balance of specific concentrations of the cytokinenes: HAP, 21P, K and the auxins: AlA, ANA, NOA. The multiplication was achieved by introducing the tissue in various

propagation circles to obtain different generations of the propagation material as follows:

Cultivation of the first tissue generation ⟶ developing the tissue and increasing its quantity ⟶ dividing the multiplied tissue to obtain the second tissue generation ⟶ cultivation of the second tissue generation ⟶ and so on (Photo No.4).

In this way, the tissue propagation material was multiplied till reaching the required quantity. This procedure may be lasted for more than one year. This was dye to adopted commercial production plan. Obtaining a tissue generation from former tissue generation, ranges between 1-2 months or more.

It is worth mentioning that the repeated cultivation produces tissue masses rapidly propagated and divided, and can always produce a new buds which grow or appear at the surface of the masses of inside them. Longitudinal sections of mature meristematic isolates showed a formation corresponding with the start of vascularization. Continuity of division cells led to vascular bundles which can be either by induced to form other independent nodules, or by the development for give arise the buds (Photo No.4).

Developing and Separating the New Buds:

the buds tissue masses were culture in a nutritive solution containing small quantities of the auxins AlA, NOA and ANA, and the cytokenines 2IP, RAP and K. This solution enhances the growth of new young buds either in the tissue masses which generates buds, or on the surface of these masses (Photo No.5).
Upon proceeding with the transfer of these tissue masses into buds, variously, developed green growths were obtained, these growths were separated and cultivated in the same nutritive solution to enhance growing and prolongation, and so on. This stage lasted 2-3 months, and at the end of it, well-developed and ready to be transferred to the rooting stage, green growths were obtained

Rooting and Offshoots Hardening:

The green growths of 2-3' leaves, 2.5 -4 cm long, were transferred to another nutritive solution to enhance rooting. AIR was added at specific concentration.

After 2-3 successive cultivation in the same solution, white swellings appeared at the bottom of the swelling stem forming the symptoms of the future roots of the in-vitro plant. The number of the main roots ranged between 1- 3 roots (and some times more than that).

It is worth noting that the root symptoms of the green growths did not appear unless the growths showed clear improvement in their development regarding increase of the leaves' number and length and the swelling at the bottom of the short stem (Photo No.6).

After finalizing formation of the in-vitro offshoots (leaves, roots and swelling at the bottom of the stem), the offshoots were transferred to a final nutritive solution, completely free from hormones.

After two successive cultivation, in-vitro plants were obtained. The plants were well-developed in respect of number of the leaves, length, formation of main strong roots which had number of primary roots and good growth of the bottom of the short stem.

Discussion and Conclusion:

In this research, green offshoots of the local varieties: Khistaoui and Zahidi, were used. It was noticed that the best time for cultivating the explants, in the basic stage, was at the dormancy stage of the plant.

This agrees with RHISS results (Personal contacts 1998 & 1999), while the minimum response was at the fruiting stage of the mother plant.

The difference in the number of the responding explants, especially in the basic stage, may be referred to the maturity degree of the explants regarding the spiral order system of the leaves, and consequently, according to their physiological status compared with some monocotyledon gramineae kinds, wheat and rise, lose their ability to respond in accordance with their maturity degree (Weknicke and Milkovits, 1984; Weknicke et al, 1981; Weknicke and Bretteli, 1980).

5-1- The Differentiation of the Pro-Meristematic Tissue:

The formation of the advantive buds involved the differentiation of new buds from tissue parts which did not normally give buds (Margara 1984, Graspar 1988).

To apply this, the lower parts of the young leaflets surrounding the top bud were removed, since they contain potential meristematic initiations in the inner epidermis lyre (Beauchesne 1997, Aissarn corn. No. 19).

The fine young leaflets were used, of which the basic parts were removed, because of their meristemic nature, consequently, the primary cultivation each time must be done by using ordinary leaf tissue explants.

The cultivation of these explants in a nutritive solution basically rich with auxines led to increasing the explants' size and the formation of the pro meristemic tissue. The auxines were used to enhance the explant's cells to reach certain level ofnon-distinction or functional specialization, and consequently, activating them physiologically and speed their division.

Using 2.4D is always important to unbind and break up the components of the tissue structure to separate cell or tissue ,groups (Margara, 1984) .Also, it is known that the auxines are important in the cell division and cell prolongation (Auge, 1984).

Using certain hormonal balance in which the percentage of the auxines is much bigger than the percentage of the cytokenines, results in unbinding and breaking up the cohesive tissue structure of the basic explant, and also in cell division, which led to important swelling in the size of the basic explants, followed by the appearance of the pro-meristematic tissue which is distinguished by white and, relatively, active tissue protrusion.

5-2- Production of the Meristematic Tissue:

The mother explants bearing the pro-meristematic tissue were transferred to a nutritive media containing high percentage of the cytokenines compared with the auxins.

The hormonal balance of the solution made, apparently, the pro meristemic tissue continue their active division which led to an important increase in the mass of this pro meristemic tissue, and consequently, developing into primitive meristemic tissue, which is characterized by its rapid division and increase in size.

It, also, can be said that the mentioned formation procedure is caused by the hormonal balance, which the explant's cells were subject to.

On one hand, it contains interior specific quantity of the auxines due to the treatment during the basic cultivation, and a quantity of the cytokenines due to the second treatment related to the formation of the meristemic tissue.

These formations may be caused by the sytokenines enhance of the multiplication of the DNA, and the chromosomes separation which encourages the cell division (Auge, 1984).

The emergence of the new bud from the primitive meristemic tissue and its transference into bud-generating-tissue, is caused by the superiority of the cytokenines' balance over the auxines', inside the explant's cells, due to the successive cultivation in solution rich with the sytokenines. The importance of the cytokenines in releasing the process of meristems and, consequently, formation, is well-known (Auge 1984), as well as the importance of the auxin and the cytokenines in enhancing the growth of the date palm buds (Amin 1984). The formation of the organs in the moncotyledonae is, generally, enhanced by a big and sudden increase in the concentration of the cytokenines (Duhoux, 1988).
On the other hand, releasing the bud formation process does not need only the combined effect of the auxins and the cytokenines, but also, the percentage of the these two kinds in the solution. Skoog 1971, mentioned the importance of the concentration of the cytokenines and the auxines, as an absolute value, and their percentage for forming the buds.

The New Technique and its Future Horizons:

We managed to specify general features, and specific features of the new technique of date palm propagation via tissue culture. This technique was applied, successfully, and regularly repeated, for more than four years all the year round for the varieties Khistaoui and Zahidi.

It is known that there are two methods for date palm propagation via tissue culture, the first is propagation via somatic embryogenesis, which is formed from granular embryonic callus, and the second is propagation via the auxiliary buds which are formed from primary top or side buds. Our new technique resembles the propagation via the advantive buds, and it depends mainly on generating new buds directly from vegetative leave parts which normally do not give any buds.

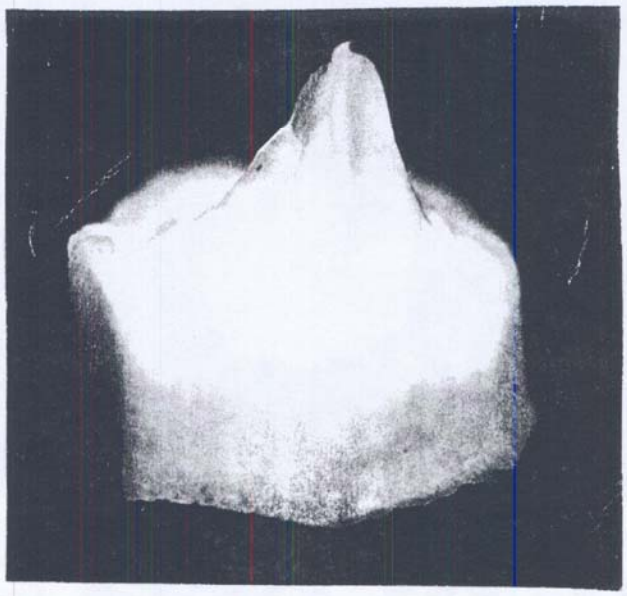
our method may be much safer in producing in-vitro plants identical to the mother plant in their vegetative specifications, compared with propagation via somatic embryogenesis, because our method does

not pass the callus stage. It is known that passing the callus stage enhances very just the occurrence of chromosomal mutation related to the number and structure of the chromosomes (Decourty, 1984). The same is applied to the propagation via somatic embryogenesis (Nasser 1996; A.O.A.D. 1995; AI wassel, 2000).

At last, we have to mention that our method does not face the serious technical troubles which the propagation via auxiliary buds suffers from, such as browning and early rooting and transparency phenomenon. Also, it is applicable all the year round, unlike propagation via auxiliary buds, which can not be applied unless in a certain specific time of the year which is at the dormancy stage of the mother plant.

Finally, it must be mentioned that this technique is being improved now to be applied at commercial level and for more than one variety.
Further, researches are to be implemented regarding:

1- study the genetic finger prints of the in-vitro plant obtained form this method, to specify the nature and percentage of the mutations, if any.
2- study the in-vitro plant's behavior in the field in order to control the phenological phase and aspects of abnormal growth, if any.



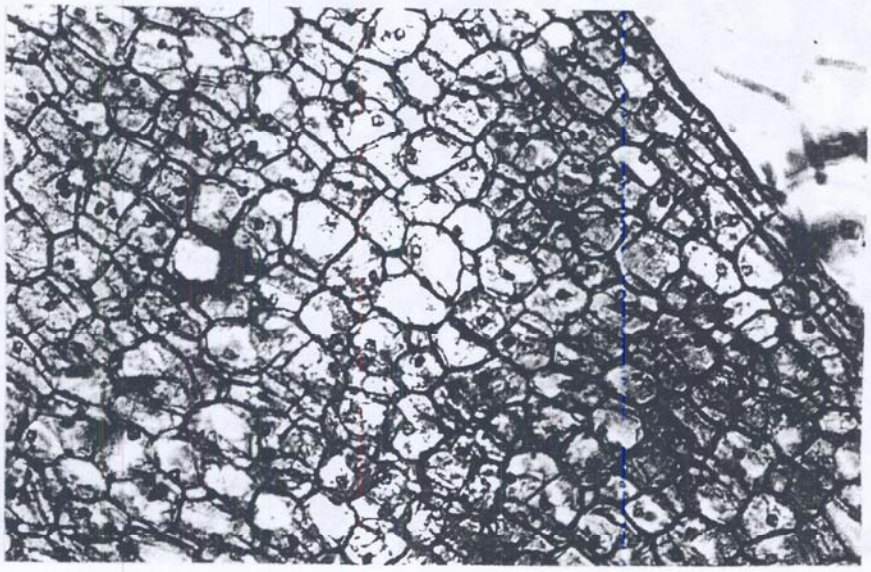
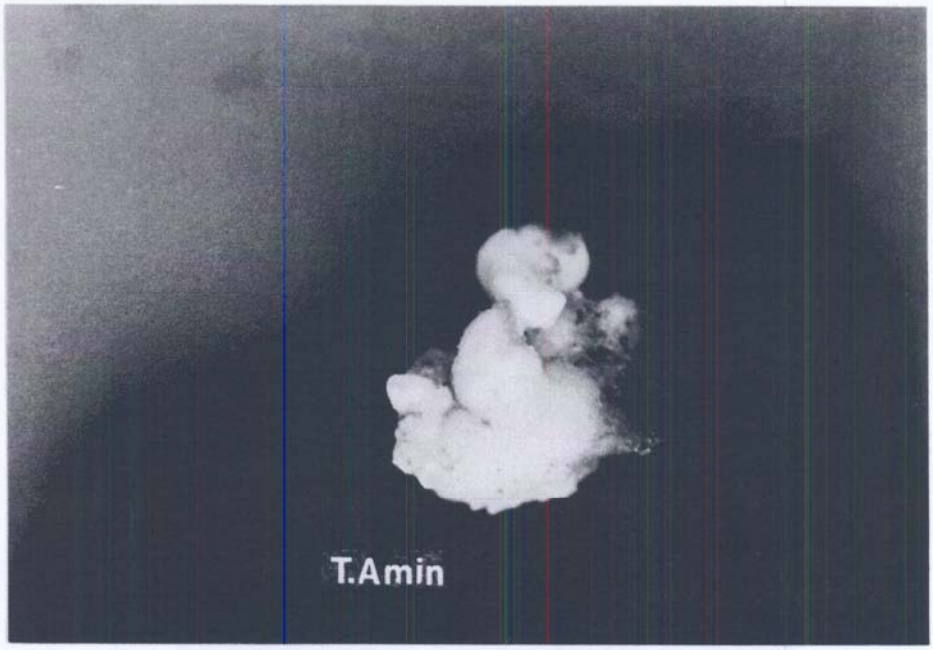


Photo No. 1 : Above , Top bud of variety Khestaoui .Below , longitudinal section of the young leaflets showed uniformed - shape tissue structure consists of small and compressed cells



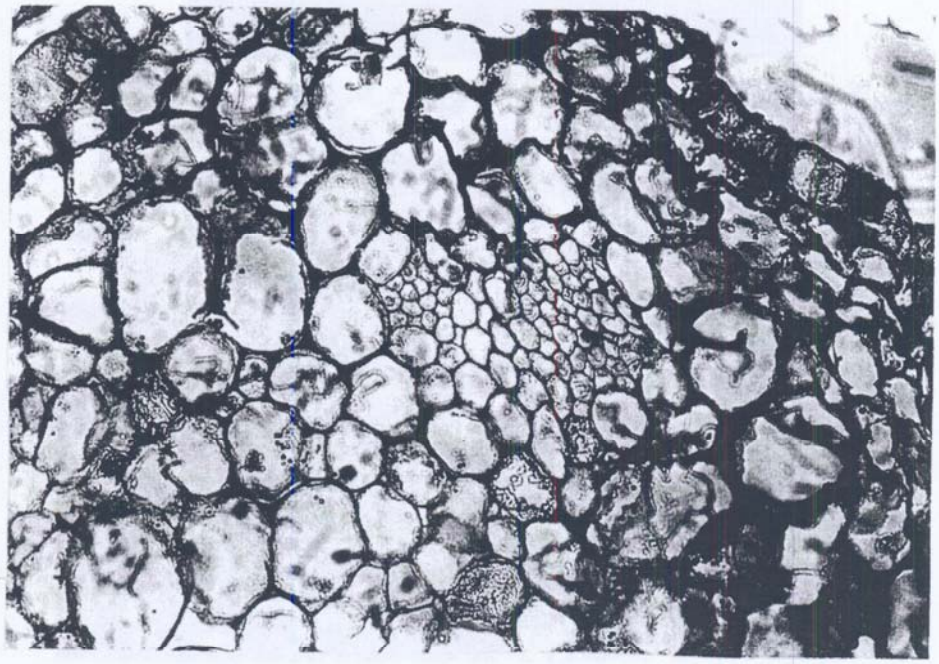
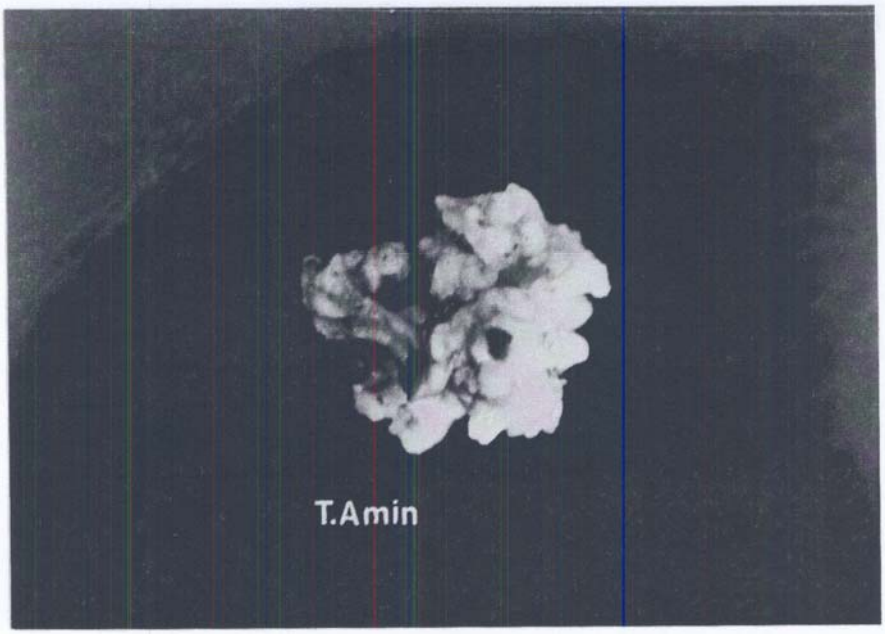


Photo No. 2 : Above , Formation of the pro – meristematic tissue on the responded explant. Below , longitudinal section of the responded explant : Fine clusters consists of very fine and active cells



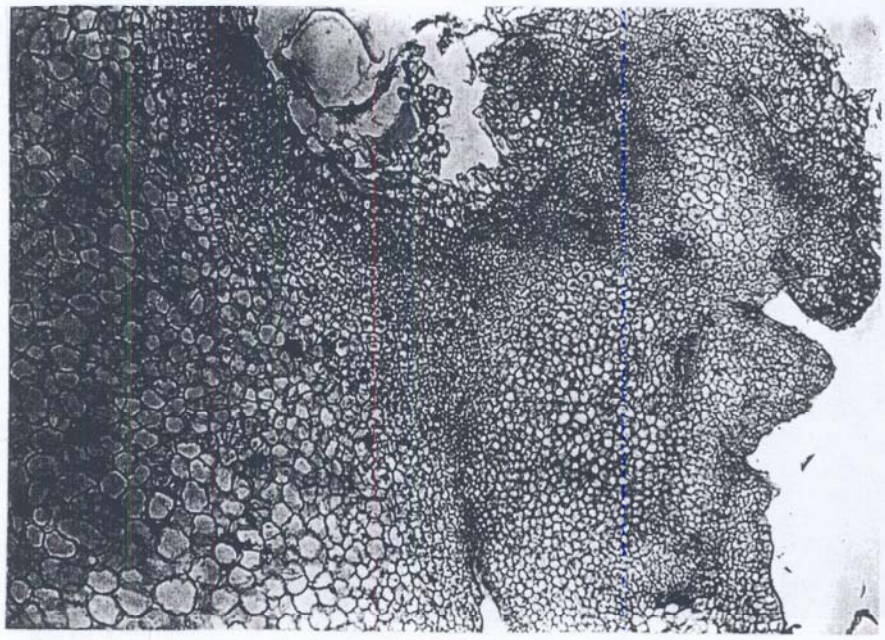
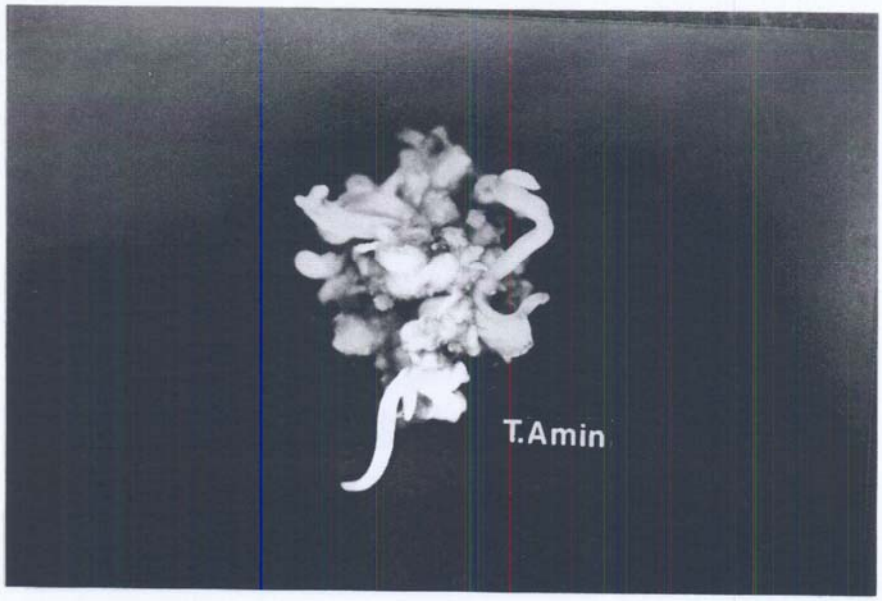


Photo No. 3 : Above , Development of pro – meristematic tissue which give arise the tissue nodes . Below , Formation of meristematic islets, which constitued by small cells .



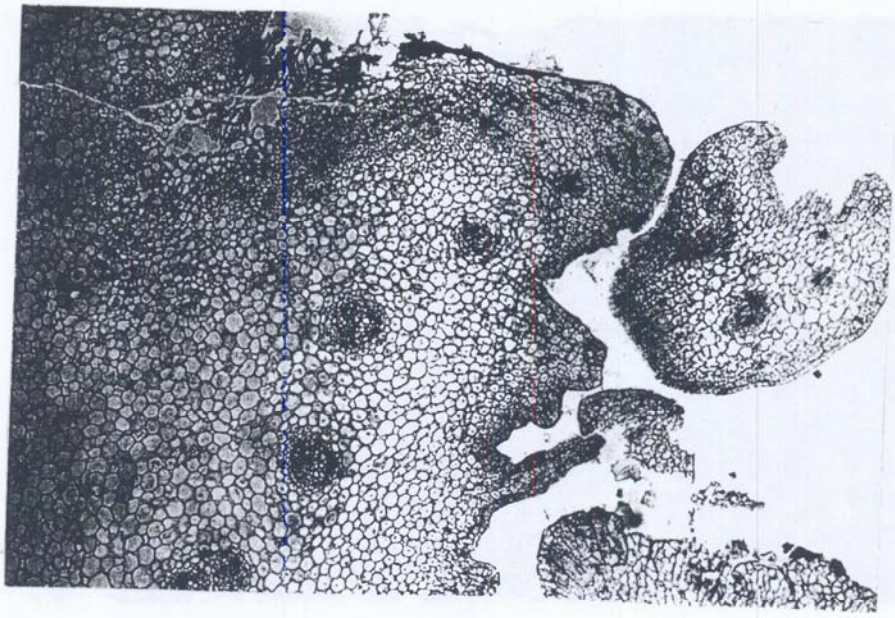


Photo No. 4 : Above , Development of propagation of tissue nodes and formation of new nodes and buds. Below , Development of meristematic tissue and Formation start of vascularization and new meristems .

582

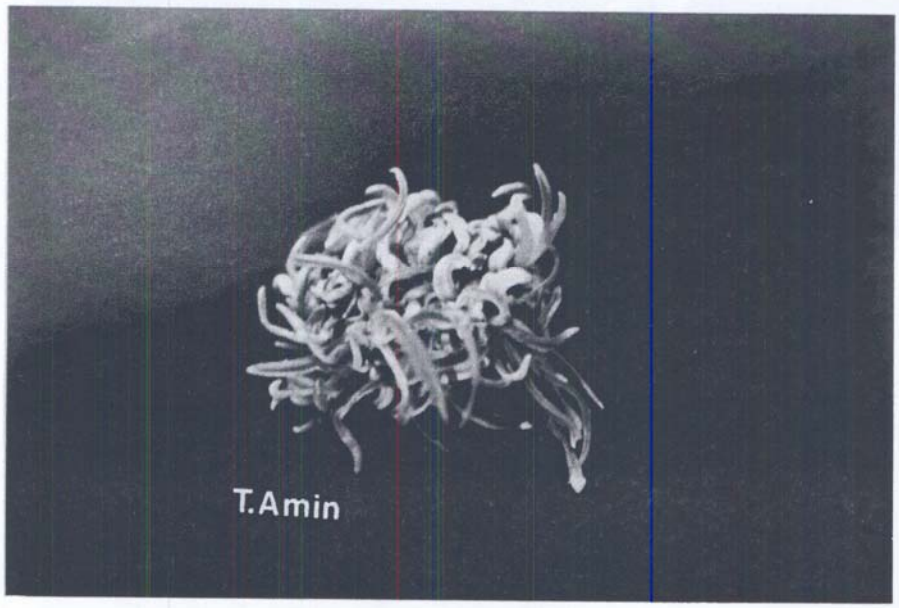
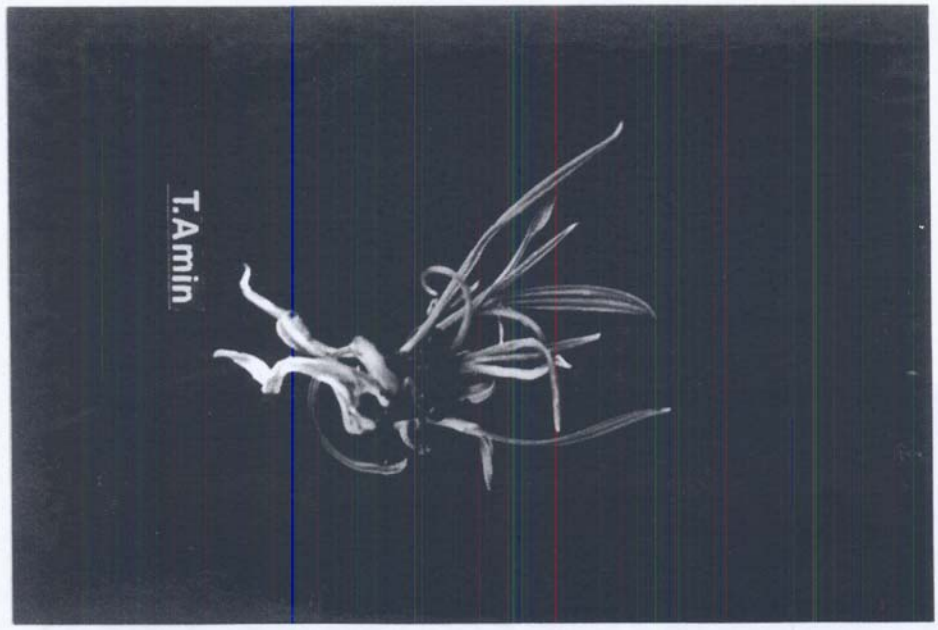


Photo No. 5 : Growth of new young buds which give arise the green growths



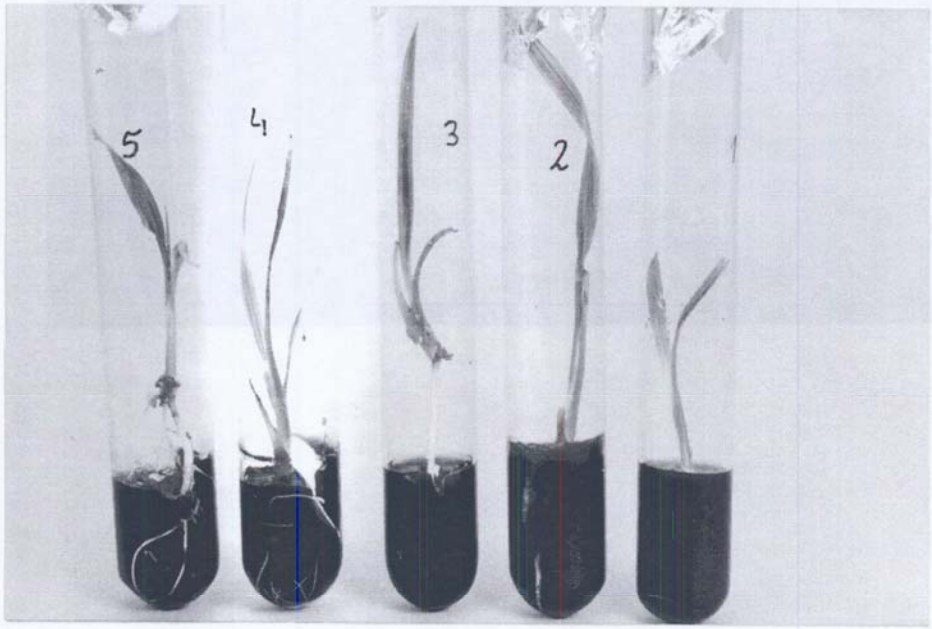


Photo No. 6 : Green growths of 2-3 leaves and formation of root symptoms and the vitro – plants .

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