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Research Paper

In vitro CULTIVATION OF *Medicago sativa* L.-A FODDER CROP THROUGH INDIRECT ORGANOGENESIS

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Abstract

The importance of the plants to mankind cannot be over emphasized. Mankind depends upon plant for food and, in many instances, for clothing, fuel, medicine, furniture and housing. It should not be surprising; therefore, that much of human endeavour has been aimed at producing and improving useful crops. Important in this endeavour has been the development of techniques for cultivating plant cells and tissue in-vitro, that is also called "Biotechnology". The objective of the present study is to regenerate *M. sativa* plants by tissue culture technique. *Medicago sativa* (Alfalfa or Lucerne) is also known as "Queen of Fodder crops", which is claimed as one of the best fodder crops throughout the world. It is one of the most important forage crops of India, grown extensively in the Rabi season. The leaves are highly nutritious, used as the animal feed and is a source of vitamin A and other nutrients. An in-vitro study was made on M. sativa using cotyledonary leaf, epicotyl, cotyledonary node with cotyledon, hypocotyl and root. Cotyledonary leaf was found to be the best responsive explant for callus initiation. It was found that in M. sativa, 80% of callus was produced after inoculating cotyledonary leaf explants on MS media supplemented with Kinetin (0.7mg/L) and 2,4-D (0.7mg/L). Out of the five types of callus from five explants, cotyledonary leaf callus was best for multiple shooting, the response was 63.3% in 18days.The MS basal medium (full strength) + 0.5mg/L IBA gives optimum response that is 58.13% for root induction in *Medicago sativa*.

Key words: *Medicago sativa, In-vitro, Cotyledonary leaf, root induction, BAP*.

INTRODUCTION

The intact plant represents a highly organized and co-ordinate system in which a large number of factors operate in the development of the whole plant with roots, stem, leaves, flowers, seeds etc. To study these factors it is necessary to cut the plant parts and grow them in different nutritional media and study the response. Thus was unknowingly born the "Tissue culture" or "*In Vitro*" culture technique. *Medicago sativa* commonly known as alfalfa or lucerne, is a perennial flowering plant cultivated as an important forage crop in many countries around the world. The name "alfalfa" comes from the Arabic language; meaning "best forage". **A**lfalfa is grown over a wide range of soil and climate conditions, it has the highest yield potential and feeding value of all perennial forage legumes. This versatile crop can be used for hay, pasture, silage, green-chop, pellets, cubes and soil improvement. Because of its many merits, especially yield, quality and versatility, it can be used successfully in many animal feeding programs. It is,

primarily used as food for dairy cattle because of its high protein content and highly digestible fiber and for increasing beef production. In recent years, tissue and cell culture techniques have been recognized as potentially valuable tool in crop improvement programmes. A short review on plant tissue culture is given below:-

A synthetic, chemically defined medium for the tissue culture of *Trifolium pretense* was developed and the media is suitable for the culture of legume species like *Medicago sativa*, *Glycin max* and *Canavalia ensiformis* and *Trifolium pretense* [7]. Tissue culture of some arboreal leguminous plants was done[11] and Plantlet regeneration was observed in *Trifolium alexandrium* [6]. Micropropagation and regeneration from callus culture was possible in *Heracleum candicans* [3]. Callus induction and organogenesis from various explants was studied in *Vigna radiat* [9]. Multiple shoot regeneration was observed from the callus culture of *Macrotyloma uniflorum* (Horse gram) [10]. The details of *in- vitro* study so far done are presented in this paper.

MATERIALS AND METHODS

Plant material and surface sterilization

Seeds are locally available in the market. The seeds were surface sterilized sequentially with 1% mercuric chloride (1 min) and finally washed in 70% ethanol (1 min). They were further washed with sterile distilled water (3×). All the operations were carried out under laminar air flow hood. Later, they were placed in conical flasks on filter paper for germination, with little sterile distilled water.

After 5 days, explants like root, epicotyl, hypocotyl, cotyledonary leaf and cotyledonary node with cotyledon were used for callus induction or regeneration. The sterilization of explants was done with mercuric chloride 0.1%, ethanol (70%) and then finally washed with sterile distilled water. The size of explants for *in vitro* experiments varies from 0.6 to1 cm each.

Culture media and culture room conditions

Various types of culture media are available for *in-vitro* experiments [6].In the present study four types of media were used like Murashige and Skoog (MS); Phillips and Collins (L2); Gamborg (B5) and Chu (N6). The media were supplemented with different hormone combinations. A critical study of these different media shows that MS is the best suitable media; therefore, this media was used for *in vitro* studies. Media was sterilized in an autoclave for 20 min at 15 lb pressure.

The media were poured in test tubes and after the slant solidified, they were inoculated with cotyledonary leaf, epicotyl, cotyledonary node with cotyledon, hypocotyl, and root. The culture tubes were kept in culture room and the photo period was maintained for 14–16 h with 2500 Lux intensity. Relative humidity was maintained at 60%. The temperature was maintained at 25 ± 2 °C. Under this controlled condition, the growth and induction of callus was studied. The response of explants was monitored on daily basis for 4 weeks.

Multiple Shooting

In the callus culture, multiple shooting is necessary for further regeneration of the juvenile plants. The multiple shoots can be excised from the callus and then sub cultured. All the five explants were inoculated on different media. The culturing process was the same as explained above. The response was monitored daily.

Rooting of Regenerated shoots

Shoots (4 – 6 cm in length) obtained from indirect organogenesis were excised from the callus and inoculated on different media in the test tube for rooting. The media is supplemented with different hormones like IAA, IBA and NAA. One medium contain only one type of hormone.

Hardening of Regenerated Plantlets

The plant lets with agar media were transferred to a sterilized beaker with about 50cc of double distilled water so that the roots are immersed in water. The plantlets were transferred to thermocol cups with a mixture of sterile sand and soil (3:1). The cup was covered with plastic bag with a few holes for air circulation. The cups were kept near the window in diffused light for 2-3 days. In 10 days the plastic cover was removed and plant was allowed to adjust to the environment. When a few new shoots are developed, the plant was transferred to non-sterile

soil with addition to water. The observation shows that it is acclimatized and then finally it is transferred to natural condition.

RESULT

Induction of Callus

As mentioned earlier five different explants were inoculated on MS medium supplemented with 0.7 mg/L Kinetin + 0.7mg/L 2, 4-D for callus initiation. For callusing the best responsive explant was found to be cotyledonary leaf. In this case 80% inoculated explant formed calli. It was followed by hypocotyl showing callus formation of 60% in 21 days. The response of callus formation of cotyledonary node with cotyledon and epicotyl was 40% and 30% respectively in 14 days. The least responsive for callusing was root which proliferated very late in 21 days and showed only 10%. Thus the cotyledonary leaf explant was found to be best responsive explant for callus formation (Table: 1 and Graph-1, Photo-1 and 2)

Different ages of seedlings (i.e. 3, 4, 5,6,7,8 days old) were removed from the germinating flask. It was found that the best responsive explant was cotyledonary leaf. This explant was excised from the seedling of different age, starting from 3-8 days. They were inoculated in the same medium (MS) with the same concentration of kinetin and 2, 4-D. The response is shown in the Table- 2 and Graph 2. It was observed that 3 days old explant did not show any response 6 days old explant showed the best response in 14 days and with 85% performance. The rest of the observations were just for academic interest.

Multiple shooing

The same five types of callus obtained from five different explants were taken and they were inoculated on MS media + 1.8mg/L BAP + 0.5mg/L 2, 4- D. Out of the five types of callus from five explants, cotyledonary leaf callus was best for multiple shooting. Its response was 63.3% in 18days. This was followed by hypocotyl with response of 38% in 21 days. Shoot induction percentage was 32% in case of cotyledonary node with cotyledon in 21 days. On the other hand 20% and 12.5% response was observed in epicotyl and root in 20 and 28 days respectively (Table 3; Photo 3 and 4).

Table 1 Showing callus formation by using different explants of *M. sativa* on MS + 0.7mg/L Kinetin +0.7mg/L 2,4-D

Types of explant	No. of explants	Response %	No. of days for response
Cotyledonary leaf	40	80%	14 days
Epicotyl	40	30%	14 days
Cotyledonary node with cotyledon	40	40%	14 days
Hypocotyl	40	60%	21 days
Root	40	10%	21 days

Table 2 Response of the age of explant (cotyledonary leaf) in formation of callus in *Medicago sativa* on MS+0.7mg/L Kinetin + 0.7 mg/L 2, 4-D.

Age of explant from seedling in days.	No. of explants inoculated.	Response %	No. of days for response
3	40	0%	0
4	40	15%	14
5	40	55%	14
6	40	85%	14
7	40	48%	21
8	40	32%	21

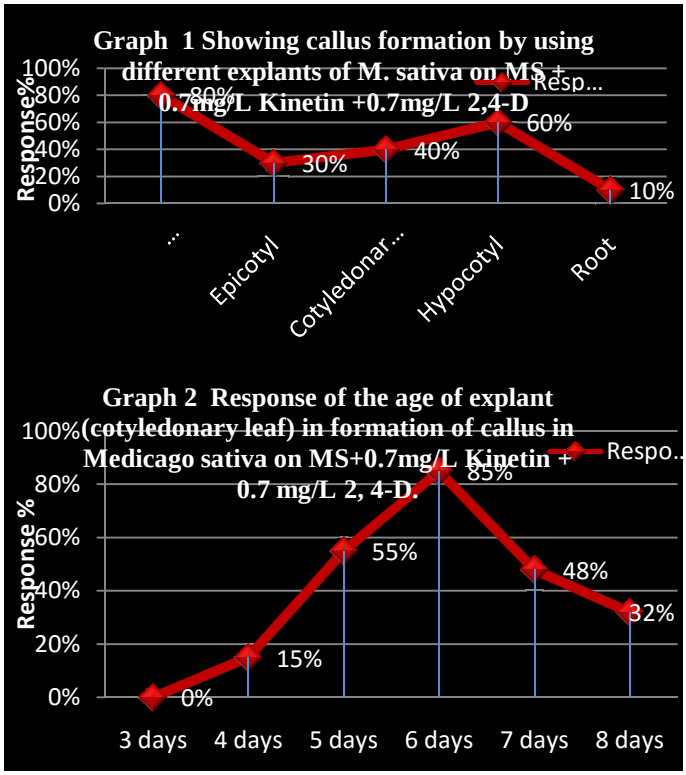

Table: 3 Multiple shoot induction in *M. sativa* on MS medium + 1.8 mg/L BAP +0.5mg/L 2, 4- D.

Calli from explants	No. of explants inoculated	% of response	No. of days for response
Cotyledonary leaf	30	63.3%	18
Epicotyl	30	20%	20
Cotyledonary node with cotyledon	30	32%	21
Hypocotyl	30	38%	21
Root	30	12.5%	28

Table 4 Root induction in *Medicago sativa* in MS media +IBA+IAA and NAA and in half and full strength of MS basal medium

MS basal medium (full strength) + IBA mg/L	% of response in root induction	MS basal medium (full strength) + IAA mg/L	% of response in root induction	MS basal medium (full strength) + NAA mg/L	% of response in root induction
0.2	6%	0.2	-	0.2	-
0.3	24%	0.3	-	0.3	-
0.4	32%	0.4	-	0.4	-
0.5	**58.13%**	0.5	11.2%	0.5	-
0.6	40%	0.6	5.8%	0.6	-
0.7	41.2%	0.7	-	0.7	-
0.8	21%	0.8	-	0.8	-
MS basal medium full strength (without hormone)				22.30%	
MS basal medium half strength (without hormone)				7.80%	

Photo.1 Induction of callus from cotyledonary leaf

Photo.2 Induction of callus from hypocotyl

Photo. 3 Subculturing of callus

Photo. 4 Initiation of shoot from callus

Photo.5 Root initiation

Photo.6 Fully developed plant

Photo. 7 Hardening of plant

Root induction

Healthy shoots were carefully transferred to rooting medium that is MS basal media (full strength) + IBA varying from 0.2mg/L to 0.8mg/L. The MS basal medium (full strength) + 0.5mg/L IBA gives optimum response that is 58.13% for root induction in *Medicago sativa* (Table 4; Photo no. 5).The response was 6% to 58.13%. When shoots were subcultured in the MS basal media (full strength) + IAA varying from 0.2mg/L to 0.8mg/L, MS basal medium (full strength) + 0.5mg/L IAA and MS basal medium (full strength) + 0.6mg/L IAA gives response that was 11.2% and 5.8% respectively. Other concentration of IAA in MS full strength basal media did not give any response. It was seen that after subculturing the healthy shoots in MS basal medium (full strength) + NAA varying from 0.2mg/L to 0.8mg/L, there was no response for root induction. When MS basal media (full strength) without hormone was used for rooting, it gives 22.30% response.

Hardening of the plants

The rooted shoots were hardened by transferring them sequentially from media to autoclaved soil and then to non-autoclaved soil. During the process of transferring the survival percentage was found to decrease. In *Medicago sativa* among 30 rooted plants 23 plants were sub-sequentially transferred to the autoclaved soil and out of 23 plants only 14 plants successfully survived in non- autoclaved soil (Photo no.6 and 7).

DISCUSSION

In the present study among four different types of media MS media shows best response and 6 days old cotyledonary leaf explant showed the best response in callusogenesis. MS media + 1.8mg/L BAP + 0.5mg/L 2, 4- D. Cotyledonary leaf callus was best for multiple shooting on MS media suplimented with 1.8mg/L BAP and0.5mg/L 2, 4- D. The response of root induction in *M. sativa* was optimum on MS basal medium (full strength) + 0.5mg/L IBA. Finally successful transplantation of plant lets were done.

In-vitro studies were done in two annual species of *Trifolium* they are *T. incarnatum and T. vesiculosu*m and 2-perenial sp. T*. repense and T. ambiguun*[7]. In that study four culture medium viz. Collins's L2 medium, Gamborg's B5 medium, Murashige and Skoog's MS media and Shenk and Hildebrandt's SH medium were used [7]. Hypocotyl was used for callus initiation [7]. Callus was easily initiated from all four *Trifolium* species on most of the media used [7].

The best of the callusogenesis takes place when the explants are in the age group of 3-10 days. It is seen in the following cases. *T. incarnatum, T. ambiguum, T. vesiculosum, T. repense* [7], 65 species of *Trifolium* [11], *Vigna radiata* [9]; *Piliostigma thonningi* [1]; *Tephrosia purpurea* [13]; *Paspalum simplex* [5], *Macrotyloma uniflorum* [10].

It is reported higher number of shoot was obtained from the meristem callus in 2-3 month [8]. On the other hand it was observed in the same material the meristem callus produces shoots in shorter period of time i.e. 2-3 weeks. The results of experiments on tetraploid *Trifolium pratense* revealed that PC-L2 to be the best medium for plant regeneration [2]..

Multiple shoot induction in *Medicago sativa* was studied[4] where 5-6 days old shoot tip were used as explants and inoculated on MS + BAP + KN. 75% tissue raised plants showed survival after transfer to soil [4]..

Proliferated shoots as well as regenerated shoots were observed from callus [10]. They were transferred to rooting medium containing auxin and combination of auxin and cytokinin [10]. A combination of IAA and KN in different concentration on MS, promoted the initiation of root at the basal region of the shoots after 20 days of culture [10].

CONCLUSION

The present study revales the regeneration protocol through effective indirect organogenesis for the fodder crop *Medicago sativa*. The protocol can be used in future for clonal multiplication and increasing the breeding activities of individual genotypes belonging to the *Medicago* species. Moreover, it is expected that the protocol could provide a potential for the genetic transformation of this important forage legume plant for improved plant characteristics.

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