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EMBRYO EXCISED CALLUS INDUCTION AND RHIZOGENESIS IN *LINUM USITATISSIMUM* L.

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ABSTRACT

Linum usitatissimum popularly known as Flax or Linseed is an economically important crop grown for food and fiber and belongs to family Linaceae. However, it has gained the interest of researchers due to its nutritional and medicinal value. In the present study, *in-vitro* rhizogenesis and callogenesis were exclusively reported from the excised embryos of Flax seeds. In the second set of the experiment, cotyledon formation and shoot induction have been achieved using incised seeds as explants. Growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) , Indole-3-acetic acid (IAA) and 6-Benzylaminopurine (BAP) alone and in combination were observed most effective. Also, the rate of callus formation has been found faster and in abundance with 8 % Sucrose in the Murashige and Skoog (MS) medium within 14 days of inoculation of the explants. Thus, the use of 8% Sucrose has proven as the most adequate energy source (Carbon) for the explants in the medium.

KEYWORDS: ***Linum usitatissimum,*** **Flaxseed, Embryo, Callus, Rhizogenesis, Sucrose**.

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INTRODUCTION

Linum usitatissimum commonly known as Flax or Linseed is an economically important crop grown in colder region of the world. It is an annual plant belonging to the family Linaceae. The plant is grown in gardens as an ornamental plant. Flax is sown and harvested much like a spring cereal crop. Although the place of origin of flax is unknown, it was likely in the near East. Flax was cultivated in ancient Egypt, where its stem fibers were used to produce linen cloth. The textiles made from flax are known in the West as linen, and traditionally used for bed sheets, underclothes and table linen. The seeds yield oil, known as linseed oil. However, flaxseed has attracted the attention of many researchers due to its potential as a 'neutraceutical' and its role as a protective and therapeutic medicinal food.[1] Research indicates several possible human health benefits associated with consumption of flax.[2] Flax seeds are the richest sources of a plant-based omega-3 fatty acids, called alpha-linolenic acid (ALA) in the world. Consumption of 3-4 tablespoons of Flax seeds everyday benefits with fiber and omega- 3 fatty acids. The omega-3 fatty acids in flaxseed promote heart, health and brain development. Besides the vitamins, minerals, fiber and protein, it contains some primary and secondary metabolites. Roots contain lignans and other phenolic substances.[3] Also, many phenolic compounds have been detected in Flaxseed.[4] Flaxseed is a rich source of natural antioxidants. With this regard, in vitro anti-cholesterol and antioxidant activity of methanolic extracts from Flax seeds has already been reported.[5] Dietary intake of polyphenols has been associated to reduce the risk of chronic diseases such as heart disease, stress induced diabetes and cancer. This is probably due to their antioxidant properties. Another unique fact about Flaxseeds is that they rank #1 source of lignans in human diets. Flaxseeds contain about 7 times as many lignans as the closest runner-up, Sesame seeds. Lignans are the phytochemicals that may play a role in preventing chronic diseases such as cancer, heart disease and osteoporosis. Flaxseed is high in fiber, but low in carbs which can support colon detoxification, fat loss and reduce sugar cravings. The alpha-linolenic acid ALA fats in flax seeds benefit the skin and hair by providing essential fats as well as B-vitamins which can help reduce dryness and flakiness. It can also improve symptoms of acne, rosacea, and eczema. This also applies to eye health as flax can reduce dry eye syndrome, lower cholesterol and weight loss. A study published in the 'Journal of Clinical Cancer Research' in the athymic mice with or without 17 β-estradiol (E2) supplementation discovered that consuming flax seeds may decrease the risk of breast cancer.[6] The three lignans found in flaxseeds can be converted by intestinal bacteria into enterolactone and enterodiol which naturally balance hormones which may be the reason that, flax seeds reduce the risk of breast cancer. Another study published in the 'Journal of Nutrition' found that, the lignans in flaxseeds may also reduce the risk of endometrial and ovarian cancer. The lignans in the flax have been shown to have benefits for menopausal women. Also, the flax seed lignin- (SDG) secolariciresinol diglucoside holds greater therapeutic potential for its application as a neutraceutical for the prevention of breast cancers.[7] Medicinally, Flaxseeds have been used in the traditional Austrian medicine internally i.e. directly soaked or as tea and externally as compresses or oil extracts for the treatment of disorders of respiratory tract, eyes, infections, cold, flu, fever, rheumatism and gout. Studies and facts mentioned above prove Linum usitatissimum as an important and interesting genus to be studied further for the extraction of its phytoconstituents and secondary metabolites from different parts of the plant and therefore the micro propagation studies using Flaxseeds as an explant have been taken into account and performed in the present work.

MATERIAL AND METHODS

(i) Preparation of Explant

The Flax seeds were purchased from the market soaked in water for 03 hours before use. Seeds were then surface sterilized with 0.1% $HgCl_2$ Mercuric Chloride solution for 5 minutes, rinsed four to five times with sterile distilled water and used for further experiment.

(ii) Culture Conditions

The MS medium was fortified with 8% sucrose and different concentrations of 2,4 - dichlorophenoxyacetic acid (2,4-D), Indole-3-acetic acid IAA and 6-Benzylaminopurine (BAP). These growth regulators were used either alone or in combination. Agar 8 % (agar agar, J. M. Vaz Pereira, Lisboa Portugal) was added to the medium as a solidifying agent. The pH was adjusted to 5.8 before autoclave. The whole set of experiment was performed in triplicate in two parts: In first part, the embryos from the seeds were excised carefully without any damage with the help of scalpel and inoculated on MS medium. Around 06-07 embryos in each jar were placed. Growth hormone concentrations Viz., 0.5, 1.0 and 2.0 mg/L of 2,4-D were added in 03 different jars. In second part, seeds with small incision were directly inoculated on MS medium aseptically. Around 10 seeds in each jar were placed. Growth hormones concentrations viz., 0.5, 1.0 and 1.5 mg/L of IAA: BAP were added in 03 different jars. The cultured bottles were transferred in an incubation room after inoculating the explants on MS medium, at 22 °C under a 16 h photoperiod. Observations were recorded after 14 days of incubation.[8,9]

RESULTS

The present study aimed to achieve convenient, cost effective and reliable method for the micro propagation of *Linum usitatissimum* with the easy availability of the seed explant. The results for both the sets of experiment have been tabulated and discussed below (Table -1). In case of first part of the experiment, where embryos were directly used, the embryogenic callus and rhizogenesis was observed dominantly (Photoplate - I. Fig-3 and 4, respectively) in 1.0 mg/L of 2,4-D concentration. The callus obtained was white and friable. Cotyledon formation was noted but with no shoot emergence (Photoplate- I. Fig-2). However, in case of second part, where seeds were inoculated directly, showed excellent results with hormonal combination of 1.5 mg/L of IAA+1.5 mg/L BAP. The callus formation , cotyledon

formation and shoot emergence was expeditiously observed (Photoplate –II,Fig.3 - 8) . The calli obtained were white and friable . On 15th day, sub-culturing of different tissues i.e. callus, roots, shoots and cotyledons was performed and maintained for future reference.

DISCUSSION

Earlier relevant studies have reported the effect of different concentrations of sucrose viz., 6% ,9% and 12 % in the modified MS medium containing 2.0 mg /L BAP + 1.0 mg /L NAA 1-Naphthalene acetic acid hormonal combination where , the influence of these sucrose concentrations was evaluated using Flax genotype cultivars, 'Lirina','Barbara', 'Mikael' and their reciprocal hybrids No. 30, No. 23, No. 31, No. 02. In this case, 9% of sucrose was reported to be significantly effective for the induction of callus in good amount.[10] In another study, the cotyledons were inoculated as explants in MS medium with 1.0 mg/L BAP combination was found to be most favorable for high frequency callus induction and the highest no. of shoot per inoculum was observed in 2 mg/L BAP**:** 0.5 mg/L NAA.[8] Also, the study on evaluation of the callus, shoot and the root formation of the hypocotyl explants was performed 28 days after the experimental setup. The highest regeneration effectiveness was observed in the media supplemented with 1 mg/l BAP or 1-Naphthaleneacetic acid NAA.[9] Whereas, in our study ,results for high frequency callus induction ,shoot emergence and rhizogenesis were prominent within 14 days of inoculation of the embryo and seed explant in the full strength MS medium. Thus, the time span to obtain profused callus was reduced in our experiment. The studies on the embryogenesis in Flax were conducted earlier where; the somatic embryos were induced indirectly from older stages of zygotic flax embryos (20–28 days old) through a callus phase. Here, for callus induction, the growth hormone 2,4-D in 2 mg/L and 5 mg /L concentrations were used.[11] The reports published on Somatic embryogenesis, organogenesis and callus growth kinetics in flax has indicated the formation and development of somatic embryos in the most efficient phytohormonal combination of 0.4 mg /L 2,4-D +1.6 mg /L Zeatin .[12] The Callogenesis in Flax has been studied using cotyledonary explants and apical nodes where 3 % Sucrose with hormones NAA = 1-Naphthaleneacetic acid, TDZ = Thidiazuron (N-phenyl-N_-1,2,3-thiadiazol-5-yl urea) ; CH = Casein enzymatic hydrolysate were proven to be most efficient.[13] The work attended in the present experiment, indicates that the MS medium supplemented with 8% sucrose concentration is most favorable medium with the additional source of energy (in the form of carbon) for the developing callus. Therefore, it is most reliable and cost effective method for the faster and convenient micro propagation of the economically, medicinally and nutritionally important plants. As far as nutritional supply in micro-propagation technique is concerned, a suitable combination of growth regulators is required to be used in the culture medium with adequate sucrose concentration ,since it is an important factor to produces desirable morphogenic responses. The easy availability of the seed explants used in the present experiment is another advantage which is devoid of the concern of viability of the explants.

Table 1

Response of experimental plant system Linum usitatissimum in varyinghormonal combinations for in -vitro regeneration.

S.N	Experimental Sets	Concentration of Growth Regulators used in mg/L	Callus Formation	Rhizogenesis	Cotyledon formation	Shoot Emergence
1	Excised Embryos placed on MS medium	0.5 (2,4-D)	+	+	-	-
2		1.0 (2,4-D)	+++	+++	+	-
3		2.0 (2,4-D)	++	++	+	-
1	Incised Seeds placed on MS medium	0.5: 0.5 (IAA:BAP)	-	-	+	+
2		1.0:1.0 (IAA:BAP)	++	-	+++	++
3		1.5:1.5 (IAA:BAP)	+++	-	+++	+++

(--) : No Response, (+) :Good, (++):Very good, (+++) : Excellent

Photoplate-I
Excised Embryos placed on MS medium


Figure 1

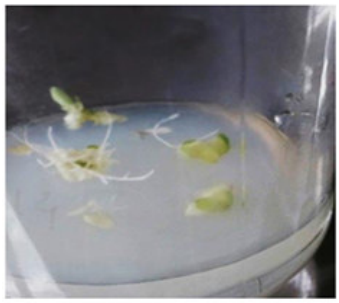
Figure 2

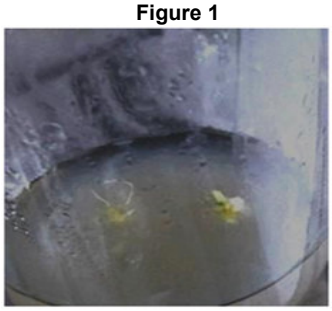
Figure 3


Figure 4

Figure 1 ***Embryos inoculated on 1st Day***
Figure 2 ***Cotyledon formation and initial rhizogenesis on 8th Day***
Figure 3 and Figure 4 ***In vitro Rhizogenesis and Callogenesis on 14th Day***

Photoplate –II
Incised Seeds placed on MS medium


Figure 1


Figure 2


Figure 3


Figure 4

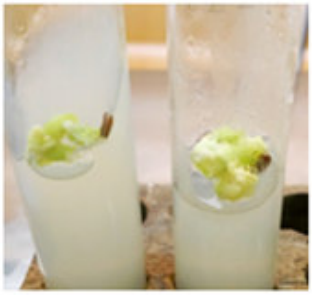
Figure 5


Figure 6

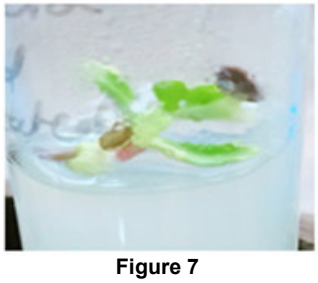
Figure 7

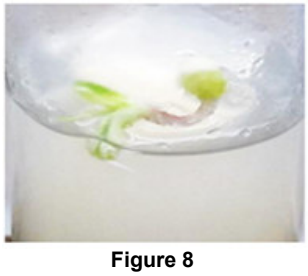
Figure 8

Figure 1 ***Incised seeds on 1St Day***
Figure 2 ***Callus initiation and Shoot emergence on 8th Day***
Figure 3 and Figure 4 ***Cotyledon Formation on 14th Day***
Figure 5 and Figure 6 ***Callogenesis on14th Day***
Figure 7 and Figure 8 ***Shoot Emergence 14th Day***

CONCLUSION

It can be concluded that, excised embryos inoculated directly on MS medium with 8 % Sucrose and 1mg/L of 2,4-D, facilitate efficient rhizogenesis and embryogenic callus formation. Whereas, in case of incised seed, 1.5 mg/L of IAA**+** 1.5 mg/L BAP combination is noted as the best suitable combination for callogenesis, cotyledon formation and shoot generation in MS medium within 14 days. It is to be noted that, for both the cases, the duration of callogenesis and cotyledon formation was shortened to 10-14 days when compared to the reports published earlier (21-28 days). This may be due to the effect of 8 % sucrose in the MS medium which might have proven to be the added source of energy (Carbon) for the explants in the medium. This finding may further help researchers in phytochemical screening and secondary metabolite elicitation from different plant tissues such as callus, root and shoot in a resourceful manner without being concerned for testing various combinations of growth regulators and choice of explants.

CONFLICT OF INTEREST

None.

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