Journal of Plant Studies; Vol. 2, No. 1; 2013
ISSN 1927-0461 E-ISSN 1927-047X
Published by Canadian Center of Science and Education

Studies on Explant Regeneration and Protoplast Culture from Hypocotyl Segments of Jute, *Corchorus capsularis* L.

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Received: March 7, 2012 Accepted: October 5, 2012 Online Published: November 15, 2012

doi:10.5539/jps.v2n1p27 URL: http://dx.doi.org/10.5539/jps.v2n1p27

Abstract

A repeatable protocol was developed for shoot regeneration from hypocotyl explants in jute (*C. capsularis*) that resulted in 25% regeneration using a combination of 6-BAP (7.5 mgl^{-1}) and adenine hemisulfate (50 mgl^{-1}). Rapid shoot proliferation was observed on medium containing NAA (0.1 mgl^{-1}) and 6-BAP (1 mgl^{-1}). The elongated shoots rooted on basal MS and could be successfully acclimatized, which later developed fertile flowers and viable seeds. After establishing regeneration protocol for jute from hypocotyl explants, the possibility of its response for protoplast isolation and culture was studied. The efficiency of isolation of protoplasts from hypocotyls was influenced by conditions of seedling growth, incubation time in enzyme solution, constitution of the enzyme mixture, osmoticum and purification methods. Isolated non-vacuolated protoplasts when cultured in modified Kao's medium with 7.2 gl^{-1} sucrose produced microcalli in about four weeks. However, these microcalli did not develop further.

Keywords: adenine hemisulfate, *Corchorus*, hypocotyl, regeneration, protoplasts, microcalli, sea water

1. Introduction

Jute is a soft, shiny, long, off-white to golden-brown and cheap natural vegetable fiber that could be spun into strong threads; produced from plants of genus *Corchorus*, belonging to the family *Malvaceae*. This 100% biodegradable and recyclable golden fiber could be successfully blended with variety of other fibers for varied environment friendly and value added end products. *C. capsularis* (white jute) is one of the two cultivated species in the genus. *In vitro* plant regeneration protocols using cotyledon and petiole attached cotyledon explants for different cultivars from both the species are well documented (Abbas et al., 1997; Islam et al., 1982; Seraj et al., 1992; Saha et al., 1999; Huda et al., 2007; Sarker et al., 2007; Pushyami et al., 2011). Jute considered recalcitrant to regeneration and the process being genotype dependent (Naher et al., 2003), different types of explants need to be tried for establishing a repeatable regeneration protocol. Plant regeneration could be achieved from cotyledonary petiole explants till now but protocols for regenerating other explants like hypocotyls and cotyledons were so far not successful.

C. capsularis and *C. olitorius* (tossa jute) have their own unique qualities. While *C. capsularis* could adapt itself to varied growing conditions and has better tolerance to water logging, *C. olitorius* possesses better yield and retting quality. However, genetic diversity in these two species has been narrowed down due to restricted selection from natural populations for quantitative traits. In addition, a strong sexual incompatibility, which causes premature cessation of embryo growth, exists between these two preventing any cross breeding (Ganesan et al., 1957; Kundu et al., 1959). Hence, creating inter-specific hybrids via natural or conventional breeding became very difficult. For the same reason, possibility of improving the fiber has become almost impossible. The only possible way to create hybrids of these species has been recognized as protoplast fusion since long (Das et al., 1981; Arangzeb & Khatun, 1983). Though there were studies on protoplast culture in cultivated jute species, none of them reported plant regeneration from protoplast derived cultures (Islam et al., 1981; Kumar et al., 1983; Saha & Sen, 1992; Khatun et al., 2002), the only exception being Saha et al. (2001) who reported a limited success in obtaining embryo like structures from protoplasts.

This background information made us initiate studies on *in vitro* regeneration and protoplast isolation and culture

from hypocotyl explants. Results reported here are with respect to white jute, *C.capsularis*.

2. Materials and Methods

2.1 Source of Explants

Seeds of the white jute cultivar JRC 698 (obtained from Central Research Institute for Jute and Allied Fibers, Kolkata, India) were surface sterilized with 70% ethanol for 1 min and 0.1 % (*w/v*) mercuric chloride (Himedia, Mumbai, India) for 2 min followed by thorough washing with sterile distilled water.

The 3-d old germinating seedlings on germination medium (MS + 1% sucrose) were transferred to light for further two days for regeneration studies and retained in dark for protoplast studies.

2.2 Regeneration Experiments

5-d old seedlings were used as explants in regeneration experiments. The cotyledons and the apical bud were excised and separated from the hypocotyl region and hypocotyl explants (1.0 cm long) were cultured on MS medium containing different combinations and concentrations of plant growth regulators (PGRs).

2.2.1 Culture Media and Culture Condition

Required PGRs were added to MS basal medium (Murashige & Skoog, 1962) and the pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. Explants were grown on medium solidified with 8.0 gl^{-1} agar (Himedia, Mumbai, India) and kept under 16 h photoperiod, illuminated with cool white fluorescent lamps at 28°C. MS basal medium was supplemented with 0.5 - 7.5 mgl^{-1} 6-BAP (6-Benzylaminopurine) in combination with 25 or 50 mgl^{-1} adenine hemi sulfate ($AdSO_4$) for callus induction and subsequent regeneration. The response was carefully monitored and scored.

Calli bearing shoot clumps were sub-cultured to medium containing 0.1 mgl^{-1} NAA and 1.0 mgl^{-1} 6-BAP for rapid shoot elongation. The number of elongated shoots was visually examined and the shoots were separated from the clump.

2.2.2 Rooting and Hardening

Elongated shoots (2–4 cm) were excised and transferred to MS medium for rooting. Individual plantlets were acclimatized in a mixture of soil and vermiculite (1:3) under culture room conditions in plastic cups covered with transparent polythene bags with holes to control humidity by gradually increasing the holes every week for 15 days. Hardened plants were transplanted into pots containing manure and soil (1:2) under green house conditions.

2.3 Protoplast Isolation

Hypocotyls and cotyledons were transversely chopped in pre-plasmolysis solution (Table 1) into fine pieces (0.05-1.0 mm thickness) in a 9 cm Petri plate. Protoplasts were isolated in a two step-stationary and gyration digestion process. Following 1h pre-plasmolysis and stationary overnight digestion at 25°C in the enzyme solution (Table 1) for cell wall degradation, they were incubated at 27°C on a rotary shaker maintained at 50 rpm for 2h. The resultant mixture was pelleted and purified over sucrose solution (Table 1). All incubations were carried in dark at 26±1°C.

2.3.1 Purification of the Protoplasts

The isolated protoplasts with debris were released by pipetting the enzyme protoplast mixture slowly through a wide bore Pasteur pipette. The enzyme solution along with protoplasts is passed through a 45 μm steel filter. Most of the cell debris got filtered at this stage. The filtrate was dispensed in a 10 ml sterile screw cap tube and centrifuged at 2000 rpm for 5 min. At this step the vacuolated protoplasts floated over the solution and formed a band that can be easily removed with a Pasteur pipette and the supernatant was discarded. To the pellet, 10 ml of 20% sucrose was added and centrifuged at 2000 rpm for 10 min again. Protoplasts form a band over the sucrose surface that could be collected in a separate sterile tube using a Pasteur pipette. The protoplasts were washed twice with 35% sea water (Table 1). At this step, protoplasts formed a thick pellet at the bottom of the tube. The protoplasts were resuspended in 4 ml of Kao's and Michayluk (1975) medium (KM) with PGRs and made into aliquots in 6 cm Petri plates to a final cell density of 1 x 10^5 cells/ml and incubated in dark at 25°C.

2.3.2 Protoplast Culture

The protoplasts were cultured in liquid medium containing varied growth regulators. Sustained divisions could be observed in KM medium containing 1.0 mgl^{-1} 2, 4-D, 0.1 mgl^{-1} NAA and 0.05 mgl^{-1} zeatin. All the growth regulators used in culture were filter sterilized using sterile 0.22 μm cellulose nitrate membrane. Dilutions of the culture media were made following the first mitotic divisions for reducing the osmotic pressure of the culture

medium.

Table 1. Solutions and culture media used for protoplast isolation and culture

Media	Composition	Method of preparation	Step where it is used
Plasmolysis solution	½ MS and 7% (*w/v*) Mannitol	pH adjusted to 5.6 autoclaved	Pre-plasmolysis
Enzyme solution	½ MS + 7 % (*w/v*) Mannitol + 1% (*w/v*)cellulose R10 (Yakult Pharmaceutical Industry Co. Ltd, Tokyo, Japan), 0.05% (*w/v*) Pectolyase Y23 (Seishin Corporation, Tokyo, Japan)	pH adjusted to 5.6 and filter sterilized using 0.2 µm filters (Sartorius)	Isolation of protoplasts
Sucrose Solution	20% (*w/v*) Sucrose + 0.05% (*w/v*) $CaCl_2$	pH adjusted to 5.6 and autoclaved	Protoplast purification
Wash solution	35% Sea water (major nutrients)	Major salts dissolved in double distilled water and autoclaved	Pelleting and washing of the protoplast pellet
Culture medium	Modified Kao's and Michayluk media (1975) along with plant growth hormones	7.2g/l Sucrose is added and pH adjusted to 5.6 and filter sterilized using 0.2 µm filters (Sartorius)	Protoplasts culture medium

3. Results and Discussion

3.1 Regeneration Experiments

Different explants were used in the past for *in vitro* regeneration of *C. capsularis* and so far cotyledons with petiole attached has been the most promising explant for this recalcitrant crop (Khatun et al., 2007). This crop is genotype dependent and fastidious about the explant type used with respect to tissue regeneration (Naher et al., 2003).

$AdSO_4$ is known to be a potent growth regulator often used in callus induction and plantlet regeneration in many plants. Purine based cytokinins-both natural and synthetic are degraded in plants to adenine and related nucleotides (McGaw et al., 1984; Forsynth & Van Staden, 1987). Despite its known activity in the adenine derived compounds (cytokinins), adenine itself is used in tissue cultures for plant regeneration. Adenine as a potent growth promoter, when used along with 6-BAP or kinetin often proved more advantageous in organogenesis of many species like *Vigna* (Ayyasami et al., 2002), jojoba (Hassan et al., 2003), pear (Yancheva et al., 2006) etc.

In the present studies, on the hypocotyl explants, callus initiated within 2-3 weeks of culture on the MS medium supplemented with 6-BAP and adenine hemisulfate. The calli obtained from different treatments could be differentiated into two types by color and texture. Type I calli obtained were soft, watery, non-morphogenic and yellow to brown in color during the early weeks of culture. The calli grew very slowly and upon subculture to medium containing 7.5 mgl^{-1} 6-BAP and 50 mgl^{-1} adenine hemisulfate, this callus showed no signs of development and browning of the explants occurred subsequently. Type II calli cultured on a medium containing 7.5 mgl^{-1} 6-BAP and 50 mgl^{-1} $AdSO_4$ were friable, cream to white in color which turned green later. The calli were compact and granular in nature. Rapid shoot proliferation could be observed in medium supplemented with NAA (0.1 mgl^{-1}), 6-BAP (1 mgl^{-1}). GA_3 at 0.5 mgl^{-1} along with 0.1 mgl^{-1} BAP was used for shoot elongation. Hypocotyls cultured on the medium containing 7.5mgl^{-1} BAP and 50 mgl^{-1} $AdSO_4$ showed a maximum response of 25% shoot bud induction when compared to other combinations (Table 2).

Table 2. Percentage shoot bud induction in hypocotyl explants in response to 6- BAP and adenine hemisulfate supplemented medium

Sl No.	BAP (mgl^{-1})	$AdSO_4$ (mgl^{-1})	% response
1	0.5	25	Callus and later browned
2		50	Callus but later brown when subcultured
3	2.5	25	10%
4		50	16.6%
5	5.0	25	11.6%
6		50	18.3%
7	7.5	25	6.6%
8		50	25%

*calculated as percentage of the number of explants that gave shoot bud induction.

3.1.1 Rooting and Hardening

Individual shoots were separated from the shoot clumps and rooted on MS basal medium with 1% Sucrose within ten days. The rooted plantlets when transferred to sterile vermiculite and incubated under culture room conditions, 100% acclimatization was observed. The acclimatized plants from the culture room conditions were carefully transferred to pots filled with soil and manure were let to grow in the green house and they established well (Figure 1) and produced viable seeds. All regenerated plants appeared normal with respect to morphology, growth and fruit set.

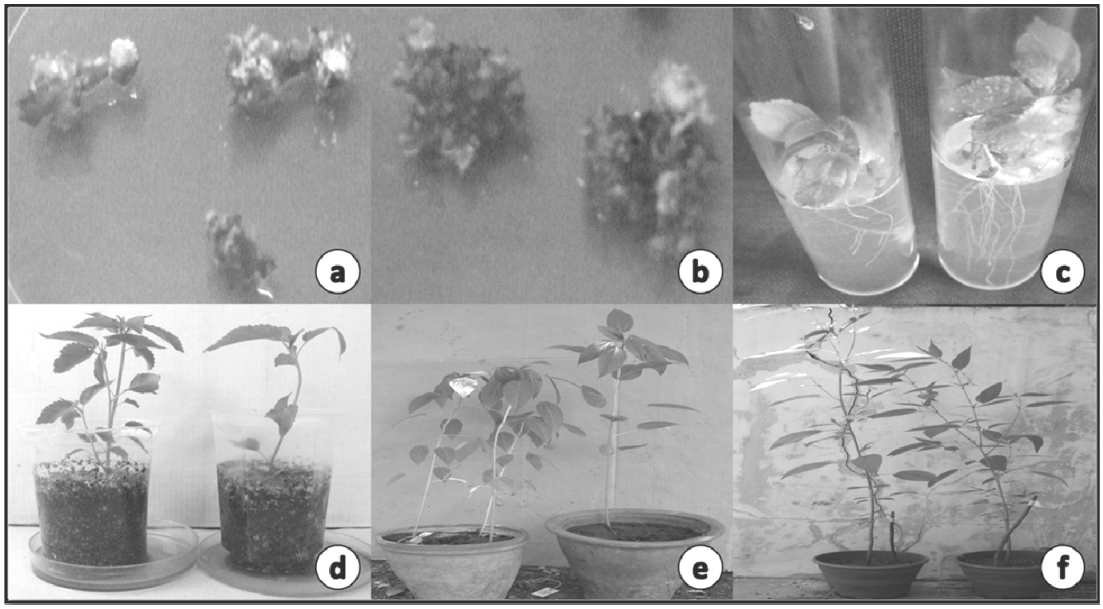

Figure 1. *In vitro* plant regeneration from hypocotyl segments of jute (*Corchorus capsularis* cv. JRC 698)

(a) Shoot bud initiation on the hypocotyl. Explant showing white compact granular callus; (b) Shoot proliferation from the callus; (c) *In vitro* rooting of the shoots on MS basal medium; (d) Acclimatized plants in culture room conditions; (e) Potted plants in green house conditions; (f) Regenerated plants that reached flowering stage.

3.2 Protoplast Isolation

Explant source has always been an important parameter while considering protoplast isolation and regeneration. Different types of explants and sources like leaf mesophyll tissues (Fu et al., 1985; Mei-Lie et al., 1987; Al-Atabee & Power, 1987; Kao & Swartz, 1987; Castelblanque et al., 2010), callus from different tissues

(Sihachkr & Ducreux, 1987; Kransnyanski et al., 1992; Chabane et al., 2010), cell suspension (Yarrow et al., 1987; He et al., 1992), hypocotyls (Newell & Luu, 1985; Chuong et al., 1987; Wright et al., 1987; Saha & Sen, 1992), and cotyledons (Saha et al., 2001; Khatun et al., 2002), were used in protoplast isolation in a variety of species including monocots for successful callus induction, regeneration, and somatic embryo development aimed at somatic hybridization and genetic manipulations. Firoozobady et al. (1986) showed that age and growth condition of the donor tissue are very important in achieving cell division and regeneration of cell wall and cell division. Suitable quantity of tissue must be taken as a starting material for obtaining good number of protoplasts as the final plating density of protoplasts is important for its further development. Since the size of the petiole explant is too small and the callus obtained from it is not enough for protoplast isolations, the other explants that can be used are hypocotyls and cotyledons, which provide good yield of protoplasts from a single experiment.

In our studies for explant standardization, cotyledons, and hypocotyls from dark germinated five day old seedlings were used for protoplast isolation and culture. Mei-Lie et al. (1987) and Hammatt et al. (1988) showed that growing the donor plants in dark for efficient protoplast isolation was important. Hypocotyl sections yielded maximum protoplast density in comparison to cotyledons and protoplast yield was less and of poor quality when younger tissues were used. Hence for further studies only five-day old hypocotyls were utilized. The yield was in an acceptable range, and debris from these preparations could be easily removed by centrifugation. In jute, there are reports of maximum yields from different explants tissues (Saha & Sen, 1992; Saha et al., 2001; Khatun et al., 2002). Hence, the protoplast yield varies considerably depending on various culture conditions and tissue sources. The present results were in accordance with the reports of Saha and Sen (1992), Saha et al. (2001), however, Khatun et al. (2002) observed that cotyledons were the best source explant for protoplast culture in *C. olitorius*. In contrast to our experiments in which we found 5-day-old seedlings to be better in *C. capsularis*, Khatun et al. (2002) have used 3-day-old seedlings to obtain the optimum results in *C. olitorius*. The use of Cellulase R10 was efficient for degrading the cell walls within a short span of time. Efficiency of Cellulase R10 was also reported by Rethmeier et al. (1991) in *Tomato*, Mathur et al. (1995) in *Arabidopsis*.

One of the major difficulties observed in jute protoplast isolation is the occurrence of high levels of mucilage in the source tissues. Similar observations were also made by Das et al. (1981), Kumar et al. (1983), and Islam (1987). As mucilage content is less in hypocotyls tissues, our experiments were continued with hypocotyl explants. To avoid mucilage secretion, the culture medium and temperature was altered during seed germination. Similar treatments were made by Khatun et al., (2002) who showed that temperature and growth conditions alter the production of certain compounds in the plant. Use of sea water for washing the explants was previously described by Kumar et al. (1983). Among the three solutions (9% mannitol, CPW salt solution (Frearson et al., 1973) and 35% sea water) used for pelleting the protoplasts in our experiments, it was observed that 35% sea water was very effective as we could get good quantity of protoplasts.

As important as explant source are the combination of enzymes used for cell lysis, protoplast precipitation solution, conditions for plating and components of the medium. The slicing of hypocotyl explants into 1mm size was optimum and yielded good quality protoplasts with one hour pre-treatment in plasmolysis solution. Among a 5% gradient of sucrose solution from 15 to 30% tried, non-vacuolated protoplasts floated and formed a distinct band over 20% sucrose solution. Our results corroborated with other studies where sucrose was used for purifying the protoplasts (Firoozabady et al., 1986; Adachi et al., 1989 etc.). High quality non-vacuolated protoplast could be extracted from the solution when 35% sea water was used. Khatun et al. (2002) found that sucrose or percoll solutions were ineffective for protoplast purification. They found 45 μm sieve a superior option.

3.2.1 Protoplast Culture

Efficient plating of protoplasts was obtained at normal culture room conditions in Kao's medium. Most of the studies on jute protoplast isolation and culture reported similar observation except for Vaz et al. (1992), who found that high molecular Oxygen atmosphere increased the chances of better plating and plant regeneration capability. For increasing plating efficiency, many groups have added different supplements into the protoplast culture medium or have altered the way the protoplasts were plated. Karamian and Ranjbar (2011) found that the plating efficiency increased up to 5 fold when nurse cells were used in *Muscari*. In the present studies, the plating efficiency was found to be at its optimum when the isolated protoplasts were cultured in modified Kao's liquid medium. At the plating efficiency of 1 x 10^5 protoplasts /ml of culture, the cell division started within five days of culture, some of the protoplasts regenerated cell walls and started dividing in 24 hours. Sustained divisions were observed after two days in medium containing 1.0 mg/l 2, 4-D, 0.1 mg/l NAA, 0.05 mg/l zeatin (Figure 2). The same protocol was tested for 25 other accessions in our laboratory but we could not obtain similar reliable results. This shows that the protoplast isolation and regeneration were purely dependent on the

genotype. Similar observations were made by Sihachkr and Ducreux (1987) in sweet potato. Micro-calli were obtained after 60 days of repeated dilutions with medium but regeneration was not obtained even on the most responsive medium standardized for *C. capsularis* (Pushyami et al., 2011) for petiole explants. Though 7.5 mgl^{-1} 6-BAP along with 50 mgl^{-1} $AdSO_4$ was proved beneficial for hypocotyl regeneration in JRC 698, no shoot regeneration could be observed even after 30 days of callus establishment in protoplast cultures. The density of culture media could be a major issue (Chuong et al., 1987; Guang-min et al., 1992). This could be another parameter to be considered while trying regeneration from callus derived from protoplast cultures.

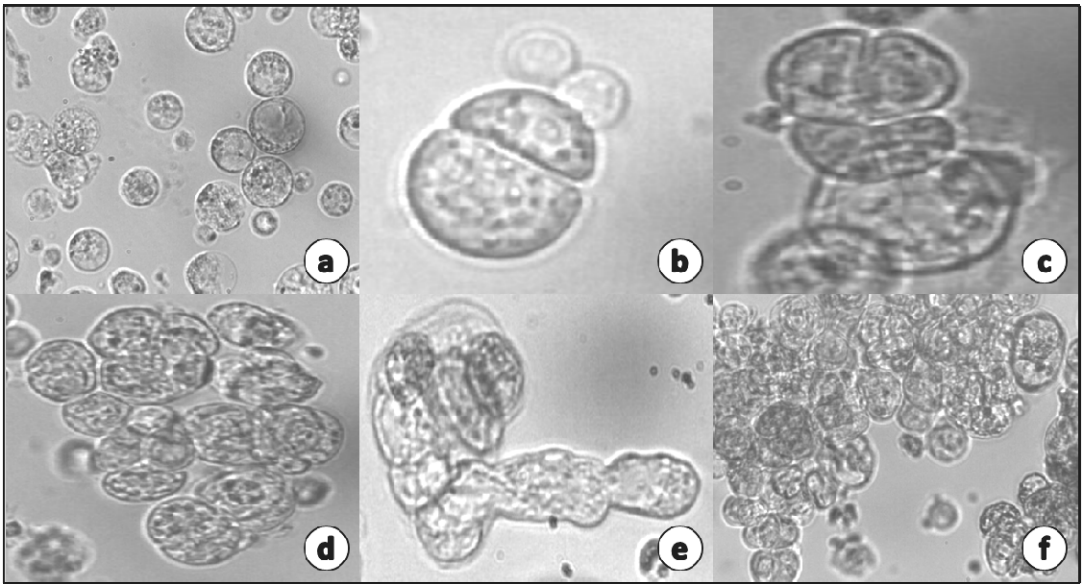

Figure 2. Protoplasts isolated from hypocotyls of Jute (*Corchorus capsularis* L.) cv. JRC 698

(a) Hypocotyl protoplasts (20x); (b) First divisions (20x); (c, d) Second divisions (4- celled stage) (20x); (e) Budding and elongation of the cultured protoplasts (20x); (f) Dividing cells forming microcalli (20x).

Expression of totipotency in protoplasts is a complex developmental phenomenon and moreover, every step in the protoplast isolation and culture induces stress to the protoplast which affects the cell wall reconstruction, cell elongation and cell cycle re-entrance (Papadakis & Roubelakis-Angelakis, 2002). Further studies must be concentrated on improvement in micro-calli production and standardization of medium for shoot regeneration from callus, so that this protocol could be utilized for further hybridization studies. Reduced anti-oxidant machinery and altered redox homeostatis are other parameters that could be considered when aiming at regeneration from protoplast cultures (Papadakis & Roubelakis-Angelakis, 2002). The increased chance of plating and regeneration capability in presence of high molecular Oxygen atmospheres as found by Vaz et al. (1992) also proves some options. It is also worthwhile to study nurse culture technique as an alternative method of microcalli culture in jute for the exploitation of protoplasts in somatic hybridization and genetic improvement of the crop.

4. Conclusion

Continuous and long history of inbreeding has caused jute cultivars to lose their genetic variability and they are strongly recalcitrant when it comes to *in vitro* manipulations. The two cultivated jute species, *C. capsularis* and *C. olitorius*, possess the distinct advantages in their own right and need to be hybridized. Exploiting the unique properties of protoplasts could create useful germplasm improvement via somatic hybrids in such sexually incompatible species. For this, a generalized and repeatable regeneration protocol is to be developed in Jute. Culturing of protoplasts poses several problems during cell culture. Mucilage, browning, lack of regenerating lines are few of the difficulties we had come across during the culture process. Although the limited progress gained from this study helps in determining some of the essential factors in isolation and culture of protoplasts in a species of white jute, further study would provide a basis for future work on the development of callus to plant regeneration protocol. The studies and the difficulties reported here could be a guideline for the future

researchers to improve their methods.

Acknowledgements

The study is supported by research grants from the Department of Biotechnology, Government of India (BT/PR/6204/AGR/16/562/2005) and Jute Technology Mission, ICAR-CRIJAF, Kolkata, India. The authors are grateful to the Head, Department of Plant Sciences for facilities under DST-FIST, UGC-CAS etc.

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