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A STUDY ON THE ISOLATION OF PROTOPLASTS FROM THE CALLUS OF *LILIUM LONGIFLORUM* OVERIG

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Abstract

Lilium longiflorum Overig is a Lilaceous plant grown for ornamental as well as certain other general purposes. The research presented here focuses on the method of protoplast isolation from the *In vitro* grown callus of *Lilium longiflorum* Overig. Series of experiments were conducted in order to optimize the conditions. Four different amounts of callus and a range of incubation time were used to achieve maximum number of viable protoplasts. However, the calli used were treated with Enzyme solution containing 0.5% (w/v) Macerozyme, 2% (w/v) Cellulase and 0.1% (w/v) Pectinase in combination, to obtain maximum number of viable protoplasts through the process of cell-wall digestion. Furthermore, an osmoticum of 20% (w/v) sucrose and a washing solution consisting of 0.1% (w/v) MES and 0.5M Mannitol were added to suspend a ring of protoplast at the interphase of both the solutions. This ring of protoplast was then isolated and tested for viability and yield. Consequently, amongst the various sets of experiment, callus of 1.5 grams and incubation time of 4 hours was found an ideal approach to obtain maximum number of viable protoplasts.

Key words: Protoplast isolation, Easter lily, Protoplast yield, Protoplast viability, Lily calli.

Introduction

Lilium longiflorum Overig, a monocot crop which is commonly known as 'Easter Lily' (Younis *et al*., 2010), belongs to the family Liliaceae, whose members are known for their prominent large flowers and unique fragrance. The genus *Lilium* in Liliaceae family comprises over 80 species and is further divided into seven sections, each having its own worth (Comber, 1949; Lighty, 1968; De Jong, 1974; Utech *et al*., 1995). The prominence of Lily in the flower industry can certainly be not undermined as it secures the fourth position in the International flower trade (Kumar *et al*.,2006), which is not just because of its ornamental value but for many other useful purposes. Lily, being a potted Easter and having a huge ornamental value, is a reason why it is mainly cultivated in the states like Michigan, California, Pennsylvania, as well as Ohio (Utech *et al*., 1995; Shry & Reiley, 2010). Furthermore, lilies are also being cultivated in the Northern Hemisphere (10^o to 60^o) including Asia, North America and Europe while Netherland stands out as the highest producer of lily amongst them (Lim *et al.,* 2008). Currently, lily occupies a significant position in the horticulture as a cut flower, potted and garden plant and ranks as the fourth most important crop in the Netherlands. Subsequently, the ability to withstand low temperatures, the outstanding shape of the flower and the pure white color that makes Lily as an attractive plant for breeding, is a reason for its increased cultivation by plant breeders (Lim *et al.,* 2008).

Although resistance to viral diseases, tolerance against unfavorable conditions for growth, potential to produce variety of flower colors and forms through conventional breeding (VanTuyl *et al.,* 1988) has introduced numerous advantageous traits but, on the contrary, it proves to be less efficient and is also very time consuming. Similarly, the incompatibility and incongruity between several different varieties of Lily greatly hinders the potential for cross combination, thereby, limiting the gene pool (Emsweller & Stuart, 1948).

In contrast, advancements in the aspect of molecular techniques has increased the efficiency of this process and has also led breeders to cross the barriers and cultivate new varieties of the plant by protoplast fusion; hence, improving the genetic make-up and enhancing the vigor of the plant. Protoplast, being the crux of plant biotechnology, serves as one of the most important tools for investigating the cell function and cell wall formation. They provide a cell system where manipulations could be achieved effortlessly by physiological and pharmacological perturbations. Experimentations of such methodology could be followed through differentiation pathways to the whole plant organism and its subsequent offspring with the desirable traits. Consequently, it is now becoming a routine practice to use protoplasts for genetic transformation, especially to study promoter strengths and in cell fusion to produce somatic hybrids (Tanaka *et al.*, 1987, Ueda *et al.*, 1990).

The founder of plant protoplast, Edward C. Cocking, presented this technology in the world of plant biotechnology with his remarkable paper on plant protoplast isolation, published in Nature in 1960 (Cocking, 1960).There are many sources of protoplasts including callus cultures, leaves, cell suspension cultures as well as preconditioned plant materials but the importance of callus-derived protoplast can be illustrated by the yield of protoplast cells obtained. Young callus cultures as compared to the older ones, are believed to be ideal for obtaining a high yield of protoplasts since the cell wall formed is not quite thick and can be easily digested by the treatment of enzymes (Razdan, 2003). Plants regenerated from cells that are isolated from cultured tissues like callus,

have the ability to retain the essential characters of a clone or cultivar (Hussain *et al*., 2013) and thereby, results in numerous protoclones (Razdan, 2003). With the first successful report on somatic hybridization in 1972 using tobacco (Carlson *et al*., 1972), it is now possible to develop the state of the art varieties through protoplasts technology for number of plants. Mii *et al.* (1994), succeeded in regenerating a fertile Lily plant from the protoplast of *Lilium* x *formolongi* by successful cell division in a solidified medium supplemented with picloram. Protoplast isolation and regeneration of cells in lilies have also been successfully reported by pollen cells that can be used for isolation and fusion studies (Tanaka *et al.*, 1987; Ueda *et al.*, 1990). However, callus cultures and meristematic cells are also being used as a starting material for protoplasts, in some lily species and cultivars (Sugiura, 1993; Mii *et al*., 1994, Godo, 1996).

Alternatively, leaves can also be used as an initiating material for protoplast isolation with species other than lilies as well (Nagy & Maliga, 1976; Binet *et al*., 1991). The regeneration potential of protoplast from lily leaves is not quite sufficient which could be one of the factors for not using them for this purpose. Perhaps, the ease of availability and handling of Lily leaf's tissues may sound less complicated and a lot more trouble-free but still only few papers on successful regeneration of leaf-derived protoplast have yet been reported (Tanimoto & Ishioka, 1994). During the past few decades, tissue culture techniques have been developed that assist and allow breeding of lily species with the desirable traits. The techniques include *In vitro* regeneration of bulblets from lily bulb scales, nodal and inter-nodal shoot tips, flowers, stems, embryos, petals as well as leaves (Kumar *et al.*, 2006). Moreover, several lily species have also been subjected to regeneration by protoplast derived from callus (Sugiura, 1993; Mii *et al*., 1994). A number of lily plants have also been regenerated from callus via organogenesis (Kanchanapoom *et al*., 2011) while several others have been regenerated by means of protoplast and some along with the nurse culture (Horita *et al*., 2002).

Different parameters were studied in the past due to the vital significance in viability testing and regeneration of protoplasts with many lily species. In order to maintain the cell division capacity and persistence of protoplasts, it is essential to consider media composition, plant growth regulators, pH, sugar type, osmotic potential, and cell density. Different enzymatic combinations with little variations have been applied for digesting cell-walls (Tanimoto & Ishioka, 1994; Godo *et al.*, 1996).

Hence, to date, this is the first protocol that presents the method of protoplast isolation from the callus of *Lilium longiflorum* Overig by means of optimization of the mass of callus and incubation time. In addition, the research demonstrated here provides an approach to obtain maximum number of viable protoplasts. The experiment giving the highest viable protoplast yield from the measured mass of callus was further tested on various sets of incubation time. Therefore, by this procedure, we were able to determine the mass of callus giving the highest protoplast yield and the optimum incubation time by which maximum number of viable protoplasts can be isolated.

Materials and Methods

Plant material and media preparation: Callus of *Lilium longiflorum* Overig was taken for the culture of protoplasts. The calli were maintained and sub-cultured on full strength MS medium (Murashige & Skoog, 1962) supplemented with 2mg/l BAP, 1mg/l IBA, 1mg/l IAA and agar. This medium serves as shooting and callus induction medium. Also, a 16/8 hours photoperiod at a temperature of 25±2^{o}C was provided to all the plants in the growth room (Fig. 3).

Effects of mass of callus on protoplast isolation: In this study, amount of calli were optimized for protoplast isolation. To identify the optimum mass of callus that influences the yield and viability of protoplasts, 0.5 grams, 1.0 grams, 1.5 grams and 2.0 grams of calli were used for protoplast isolation.

Treatment with enzyme solution: Each set of calli was suspended in 7ml of enzyme solution containing 2% (w/v) Cellulase, 0.1% (w/v) Pectinase and 0.5% (w/v) Macerozyme at pH 5.8. Suspensions of calli were then incubated in a shaking incubator at 45rpm for 4 hours in dark at 25±2ºC.

Isolation of protoplasts: After the cell wall digestion, the cells and enzyme mixture was filtered through a 45µm nylon sieve followed by centrifugation for 5 minutes at 750rpm. The pellets obtained were suspended in a washing solution and centrifuged twice at 750 rpm for 4 minutes each. 1.5ml volume of washing solution was again added to the pellet and then layered onto 20% (w/v) sucrose gradient. This mixture was centrifuged at 300 rpm for 4 minutes to obtain a ring of protoplasts at the interphase. The fraction of protoplast layer was gently removed with the help of a Pasteur pipette and transferred to another tube. The volume of protoplast obtained was further divided into 2 tubes, one for the determination of yield and viability, and another for the regeneration of plants. All experiments were conducted in triplicates.

Effects of incubation time on protoplast isolation: The time required to breakdown the cell wall by the enzyme solution, is known as the incubation period of protoplast isolation for that plant. To investigate the optimum time required to obtain the maximum yield of protoplasts, the calli samples were incubated with enzyme solution at various incubation duration i.e. 2 hours, 4 hours and 6 hours.

Yield testing of protoplasts: The isolated protoplasts were observed under compound microscope and counted using a haemocytometer. The protoplasts were viewed at 40X magnification and numbers of protoplasts observed were recorded. Yields of protoplasts were calculated using the equation given below (Tee *et al.*, 2010).

Protoplast Yield = Total cell count × Total volume of suspension/ 4 × Weight of fresh tissues (g)

Viability testing of protoplasts: The viability of protoplasts was monitored using Fluorescein diacetate (FDA) with a final concentration of 5mg/ml (Nadel, 1989). Images were taken through fluorescent microscope Nikon TE 2000E.

Regeneration of callus: Half of the protoplasts were cultured on modified MS medium (which contains ammonium nitrate at 1/8 of its normal strength) supplemented with 4.1µM picloram (Horita *et al.*, 2002). The samples were sub-cultured every week by the addition of approximately 2ml of modified MS medium.

Results

Protoplast yield with respect to the amount of callus: In order to determine the yield of the protoplast we conducted four sets of experiments, using 0.5, 1.0, 1.5, and 2.0 grams of callus and incubated for 4 hours. When observed through haemocytometer, 1.5 grams of callus showed the greatest yield of protoplast (7.28×10^4 protoplasts/ g FW). 2 grams of callus gave the lowest yield of protoplast, (2.16×10^4 protoplasts/g FW) and 1.0 gram shown a moderate protoplast yield (5.95×10^4 protoplasts/ g FW) as shown in (Fig. 1). Although, a near-maximum protoplast yield was observed in a sample containing 0.5 grams of callus that was about 7.1×10^4 protoplasts/ g FW (Fig. 1).

The viability test with FDA was showing the maximum result with a sample containing 1.5 grams of callus (Fig. 4c). However, approximately 99% of the viable cells were observed in a sample containing 0.5 grams of callus (Fig. 4a) but since the yield of this set of experiment was low, it cannot be considered as the best one. On the contrary, an average number of viable cells were observed in a sample containing 1.0 and 2.0 grams of Lily callus (Figs. 4b and 4d).

Protoplast yield with respect to the incubation time: Four sets of experiments were conducted to determine the yield of protoplasts with respect to the incubation time i.e. 0 hours, 2 hours, 4 hours and 6 hours. Incubation time of 4 hours had shown the best protoplast yield of about 3.89×10^5 protoplasts/g FW, and the lowest yield of about 0.67×10^4 protoplasts/g FW at 0 hours of incubation as shown in (Figs. 2 and 7). However, yield below than average, 7.5×10^4 was obtained at 2 hours of incubation. In contrast, a moderate yield of protoplast was observed at 6 hours of incubation with a value of 2.27×10^5 protoplasts/ g FW (Figs. 2 and 7).

Despite the yield production of protoplasts, the viability testing of each set of experiment showed quite similar results. Set 1 which was not incubated at all, showed only one to two viable cells (Fig. 6a). Set 2 that was incubated for 2 hours, showed low protoplast yield, containing a low percentage of viable protoplasts when observed through bright field of fluorescent microscope (Nikon TE 2000E) as shown in Fig. 6b. Set 3 was incubated for 4 hours, showed the highest yield and observed to be containing the greatest number of viable protoplasts (Fig. 6c). However, a moderate number of viable cells were observed in a sample, which was incubated for 2 more hours i.e., 6 hours even after having an average protoplast yield as shown in (Fig. 6d). Hence, set 3 have the best result amongst the rest as it was seen to be containing an ample number of viable cells along with the optimum yield of protoplasts.

Discussion

In this study, a protocol was established to determine the optimum conditions needed to isolate a sufficient number of viable protoplasts from the callus of *Lilium longiflorum* Overig. Results of the experiment showed that when determining the protoplasts yield against the amount of callus used, it was observed that 1.0 and 2.0 grams of callus of *Lilium longiflorum* Overig were proved to be inefficient as the yield obtained was as low as 5.95×10^4 and 2.16×10^4 protoplasts/ g FW respectively (Fig. 1). Experiment conducted with 0.5 grams of callus showed a great percentage of viable cells but since the yield obtained was not sufficient, this set of experiment was not chosen to be the best one. Set 2, containing 1.5 grams of callus showed a high percentage of cell's viability along with the highest yield of 7.28×10^4 protoplasts/ g FW (Fig. 1). Therefore, Set 3 of the first experiment and Set 2 of the second experiment provide the ideal parameters to obtain the maximum number of viable protoplasts.

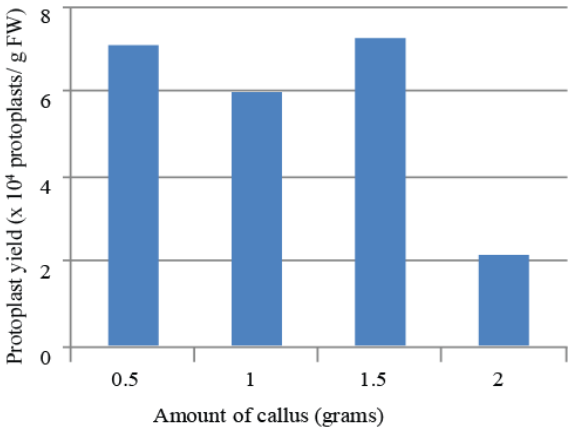

Fig. 1. Effect of different amounts of callus on protoplast yield from the callus of *Lilium longiflorum* Overig.

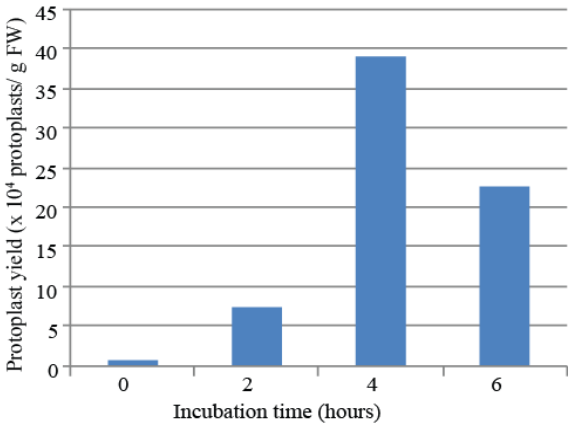

Fig. 2. Effect of different incubation times on protoplast yield from the callus of *Lilium longiflorum* Overig.

Fig. 3. (a) Callus of Lilium longiflorum overig grown on induction medium. (b) Callus finely chopped before suspension into the enzyme solution. (c) Callus incubated in enzyme solution. (d) Protoplast ring at the interface of a gradient.

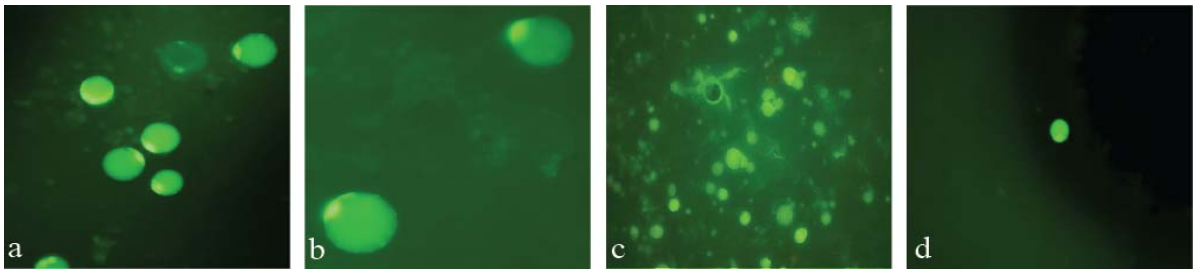

Fig. 4. (a) Viability of protoplasts isolated from 0.5 grams of callus. (b) Viability of protoplasts isolated from 1.0 grams of callus. (c) Viability of protoplasts isolated from 1.5 grams of callus. (d) Viability of protoplasts isolated from 2.0 grams of callus.

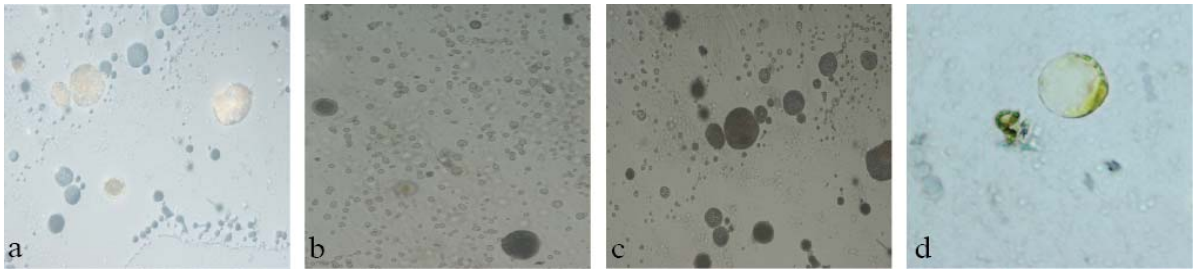

Fig. 5. (a) Yield of protoplasts isolated from 0.5 grams of callus. (b) Yield of protoplasts isolated from 1.0 grams of callus. (c) Yield of protoplasts isolated from 1.5 grams of callus. (d) Yield of protoplasts isolated from 2.0 grams of callus.

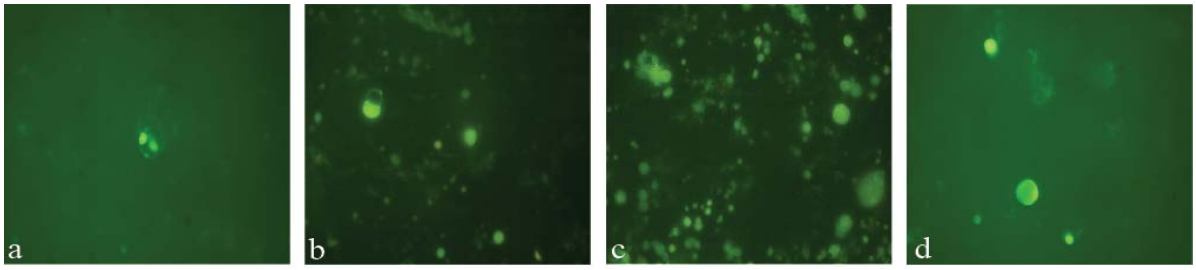

Fig. 6. (a) Viability of protoplast isolated at 0 hours of incubation. (b) Viability of protoplasts isolated at 2 hours of incubation. (c) Viability of protoplasts isolated at 4 hours of incubation. (d) Viability of protoplasts isolated at 6 hours of incubation.

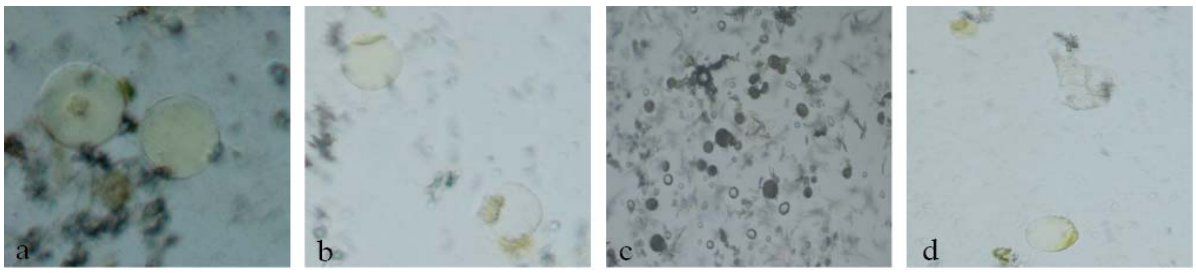

Fig. 7 (a) Yield of protoplast isolated at 0 hours of incubation. (b) Yield of protoplasts isolated at 2 hours of incubation. (c) Yield of protoplasts isolated at 4 hours of incubation. (d) Yield of protoplasts isolated at 6 hours of incubation.

Furthermore, when determining the protoplast yield against the enzyme incubation time it was found that Set 2, which was incubated for 2 hours, allowed the removal of cell wall of only few cells as the time for complete digestion was insufficient. Therefore, low number of viable protoplasts was obtained after 2 hours of incubation (Fig. 2). In contrast, Set 4, incubated for 6 hours was seen to be containing an average number of viable protoplasts since the culture was turned brown and many viable cells may have died by then (Fig. 2). Also, the control set that was not incubated at all and conducted at 0 hour of the experiment, observed to be containing only 0.67×10^4 protoplasts/ g FW, which is the lowest protoplast yield isolated and only because of the osmotic pressure. Finally, Set 3, that was incubated for a moderate limit i.e, 4 hours, callus did not turn brown at all even provided enough time for the enzyme solution to digest the cell wall of large number of cells; thereby, providing the highest yield of viable protoplasts which is 38.9×10^4 protoplasts/ g FW (Fig. 2).

The ring of the protoplast obtained at the interphase of osmoticum was observed to be light green in color and when stained with FDA (Nadel, 1989), the viable and green fluorescing protoplast observed through fluorescent microscope appeared to be quite large and the organelles were seen to be concentrated at a point along the cell membrane. Thus, the protoplasts isolated from the callus of *Lilium longiflorum* Overig were seen to be 'eye-shaped' (Fig. 3d, 4b). Besides, the results according to our experiment i.e. mass of 1.5 grams of callus with the incubation of 4 hours proved to be an ideal time period in order to obtain the maximum yield of viable protoplasts; thereby, more possibility of regeneration of plant on the medium. Isolated protoplasts in the Petri plates were observed and sub cultured after every one week by the addition of approximately 2ml of fresh modified MS medium (Horita *et al.*, 2002). The increase in the density and the growth of suspended protoplasts clearly indicated its regenerative ability and its potential for transition into a plant.

The novelty of this experiment is that no work has yet been published on *Lilium longiflorum* Overig. However, some experiments were conducted on other related species. The results proposed in these experiments are undoubtedly supported by those of Horita *et al.*, (2002), as 1.0 gram of callus was incubated in the same enzyme solution for 2 hours but they worked on only one set of experiment. However, this study presents a range of incubation times as well as the mass of callus, through which another improved and optimized approach is obtained, that is the incubation of 4 hours and the mass of callus as 1.5 grams.

(Tanaka, 1988) also worked on the same principle that is the isolation of protoplast from *Lilium longiflorum*, observed the protoplasts to be completely spherical in shape and fluorescing bright green when stained with FDA. The organelles observed in those protoplasts were also as clear as seen in this study, but were not seen to be concentrated at a point along the cell membrane. Although, this distinction in the shape of the protoplasts may be due to the fact that the explant chosen for the experiment was different (friable green calli) as compared to previous reports like Tanaka, (1988) where pollen of the plants were used as the starting material for the protoplast isolation. In addition, Lily pollen protoplasts have also been thoroughly studied by other researchers that includes (Hiroshige *et al*., 1988), they reported the isolated and cultured protoplasts derived from same plant, i.e., *Lilium longiflorum.*

The main plant growth regulators such as auxin and cytokinin, alone or in combination, are generally essential for efficient protoplast division in plant systems (Davey *et al*., 2005; Ahmad *et al*., 2011). Plant growth regulator concentrations and combinations need to be optimized for each protoplast development step (Abbasi *et al*., 2011). Moreover, in conformity with the results of Kamo & Han (2012), they proposed that the regeneration of callus of *Lilium longiflorum 'Nellie White'* was optimum when cultured on MS medium and Picloram. The cultivar of this experiment is however, different, yet the findings are in accordance with the results as the protoplast when cultured on modified MS medium (Horita *et al.*, 2002) along with Picloram, initiated the regeneration of callus successfully. The potential for regeneration was clearly observed by the dense population of callus-inducing cells (Khan *et al*., 2014).

In conclusion, the data reported here demonstrate for the first time, the optimized conditions and the parameters to isolate the protoplasts from the callus of *Lilium longiflorum* Overig to further regenerate the plants on modified MS medium (Horita *et al*., 2002) supplemented with 4.1µM Picloram. This effective approach offers the ability and the possibility to obtain maximum yield of protoplast with highest number of viable cells.

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