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PEG-induced fusion of phosphatidylcholine-liposomes with protoplasts and post-fusion evaluation of plating efficiency and enrichment in plasmamembrane phosphatidylcholine of protoplasts in *Datura innoxia* Mill

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Liposomes entrapping fluorescein diacetate were fused with protoplasts of *Datura innoxia* Mill by employing polyethylene glycol (PEG) as the fusogen. Factors that influence liposome-protoplast fusion were optimized as a function of PEG-concentration and incubation duration, liposome composition and surface charge and liposome:protoplast ratio. Phosphatidylcholine-liposomes were found ideal for the objectives of the study. Fusion index based on per cent fluorescing protoplasts varied among the protoplast types. PEG-incubation duration in the fusion assay and growth ability of protoplasts to form microcalli subsequent to liposome-protoplast fusion was determined based on protoplast plating-efficiency. Plating efficiency of post-fusion protoplasts increased due to incorporation of liposome-phosphatidylcholine in the plasmamembrane of protoplasts. Results are discussed in relation to the application of liposome-protoplast fusion system in selective modification of plasmamembrane phospholipids of protoplasts.

Keywords: *Datura innoxia* Mill, Liposomes, PEG, Phosphatidylcholine, Plasmamembrane phospholipids, Plating efficiency, Protoplasts

Liposomes are widely employed to deliver entrapped macromolecules such as DNA, RNA, antisense oligonucleotides and enzymes and small molecules such as ions, antibiotics and drugs, into animal cells and plant protoplasts[1-4]. DNA delivery by liposomes into plant protoplasts is an ideal method for genetic transformation and raising trangenics, especially for protecting the foreign DNA from DNase. Even though culturing protoplasts is difficult in many crop plants such as cereals and legumes, Solanaceae members are easily amenable[5]. Induction of desired *in vitro* responses by employing protoplast methodologies suffer from several inherent metabolic deficiencies in the protoplasts such as reduced activity of antioxidant machinery and accumulation of cytotoxic biomolecules, e.g. methyl glyoxal[5,6].

Besides serving as carrier for entrapped substances for their delivery into protoplasts, liposomes might find applications in specific manipulation of plasmamembrane lipids by liposome-protoplast fusion wherein constituent lipids of liposomes, e.g. phospholipids, could be incorporated in the plasmamembrane of recipient protoplasts[1,7,8]. When employing liposome-protoplast fusion system for the manipulation of plasmamembrane phospholipids, the incorporated phospholipids might have only a short half-life in the plasmamembrane[8]. Rapid responses that occur in a few minutes in relation to change in the turnover of phospholipids have been shown to be functionally significant in many plant systems[9,10]. Thus, manipulation of plasmamembrane phospholipids *via* liposome-protoplast fusion could offer a possibility to study the *in vitro* responses of protoplasts in a modified membrane-lipid milieu[7].

Manipulation of membrane-lipid composition in intact plants and in cultures by supplementation of lipid precursors such as choline chloride, ethanolamine and tween esters of fatty acids have been reported[11-14]. Yamada *et al*.[8] have reported phospholipid composition of products of protoplast fusion and highlighted its functional significance in the growth of fusion product. However, no report is

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Abbreviations: DP—dicetyl phosphate; FDA—fluorescein diacetate; Kn-kinetin; ML—multilamellar; PA—phosphatidicacid; PC—phosphatidylcholine; PE—phosphatidylethanolamine; PG—phosphatidylglycerol; PI—phosphatidylinositol; PMEF—plasmamembrane-enriched fraction; PS—phosphatidylserine; SA—sterylamine; Spd—spermidine; SS—stigmasterol; UL—unilamellar

available on the manipulation of plasmamembrane phospholipids by employing liposome-protoplast fusion system in relation to the elucidation of functional role of phospholipids.

In the present study, following aspects were examined– (a) parameters that influence liposome-protoplast fusion; (b) frequency of fusion of liposomes with different protoplast types; (c) plating efficiency of post-fusion protoplasts; and (d) evaluation of enrichment of liposome-phospholipid in the plasmamembrane of post-fusion protoplasts in order to assess its incorporation. This paper reports for the first time that fusion of liposomes with protoplasts resulted in increased growth ability of post-fusion protoplasts.

Materials and Methods

Plant materials and growth conditions—Leaves of the third node from the apical end of nursery-grown plants or leaf-derived callus cultures of buckweed, *Datura innoxia* Mill were employed. Whitish callus cultures were initiated and maintained in B5 + NAA (10^{-6} *M*; Fig. 1A) [15]. Whitish callus was transferred to B5 + NAA (10^{-6} *M*) + kinetin (Kn; 10^{-6} *M*) and B5 + NAA (10^{-6} *M*) + Kn (10^{-5} *M*) for inducing greening and shoot differentiation responses, respectively[16]. Protoplasts prepared from different calli, viz., whitish and green, and green region and nodules of shoot-differentiating callus and leaf were employed. Callus- and protoplast- cultures were maintained in a culture room at 25° ± 1°C, 80% RH, 16 h light:8 h dark under white light from cool white fluorescent lamps at 1200 μWcm^{-2}.

Protoplast isolation—Protoplasts were isolated from different calli and leaf as described earlier[17]. For liposome-protoplast fusion assay (hereafter referred as fusion assay) protoplast pellet was washed (3x) and resuspended in 0.6 *M* of D-mannitol solution containing 10 m*M*, $CaCl_2$; and 50 m*M*, phosphate buffer (*p*H 7.0)[18]. For preparing protoplasts for cultures washing was done with the culture medium. Protoplast density was determined through haemocytometer.

Protoplast cultures—Protoplasts were cultured in B5 + NAA (10^{-6} *M*) + Kn (10^{-5} *M*) + spermidine (Spd; 5 m*M*) containing 0.6 *M* mannitol and 10 m*M* Mes at *p*H 5.6 by layering the protoplast suspension on Phytagel (0.2%) based semi-solid nutrient medium (40 ml) in 90 mm petridish. In all the experiments, a constant inoculum (ca. 1.25×10^4 protoplasts) in 0.8 ml of protoplast culture medium was dispensed centrally on semi-solid nutrient medium and spread uniformly by using a cell spreader.

Protoplast plating efficiency—Microcalli formed in different protoplast cultures were scored on 23rd day subsequent to plating by using a colony counter (Fig. 1B). Per cent plating efficiency was calculated based on the number of microcalli formed and the total number of protoplasts plated.

Protoplast viability—This was determined based on fluorescein diacetate (FDA)-fluorescence[7].

Liposome preparation—Liposomes were prepared according to the procedure of Hub and Zimmeramann[19]. Phosphatidylcholine (PC; 20 mg; 25.3 μmole) either alone or in combinations with stigmasterol (SS; 10.4 mg; 25.3 μmole), sterylamine (SA; 1.94 mg; 7.2 μmole) or dicetyl phosphate (DP; 3.94 mg; 7.2 μmole) was employed. Molar ratio of constituent lipids in different liposomes was as follows– PC:SS-7:7; PC:SA-7:2; PC:SS:SA-7:7:2; PC:DP-7:2; PC:SS:DP-7:7:2. PC content was kept constant in the different liposomes. Liposomes were prepared in 4 ml of 0.6 *M* mannitol solution containing 0.01% FDA and 50 m*M* phosphate buffer at *p*H 7.0. Liposomes were washed (3×) and suspended in 1 ml of 0.6 *M* mannitol solution containing 50 m*M* phosphate buffer at *p*H 7.0. Liposome density was determined by employing a mixed suspension of protoplasts and liposomes wherein protoplast density was known and relative distribution of liposomes was scored in relation to protoplasts in phase-contrast microscopy field[20,21]. PC (20 mg) yielded ca. 6×10^8 liposomes.

Fusion assay—Fusion assay was performed in Silane (BRL, USA) washed, screw capped, flat bottom, glass vials (6 cm height × 2.6 cm internal diam). Assay mixture in 0.8 ml contained specified type of protoplasts at a constant amount (2.4×10^6 protoplasts), varying composition/density of FDA-entrapped liposomes, PEG-6000 (1% w/v; unless otherwise specified), mannitol (0.6 *M*) and phosphate buffer (50 m*M*) at *p*H 7.0. The assay mixture was incubated at 25°C for 1 h in a rotary shaker at 30 rpm. Immediately after incubation, the suspension was diluted with mannitol solution (0.6 *M*) containing 10 m*M* $CaCl_2$, 10 m*M* Mes at *p*H 5.6 and the protoplasts were pelleted down by centrifugation at 150×*g* for 2 min. The pellet was washed (3×) and suspended in 0.6 *M* mannitol solution containing 10 m*M* $CaCl_2$ and 10 m*M* Mes at *p*H 5.6. Aliquots of

the suspension were used for microscopy. Typically, a complete fusion assay contained FDA (0.01%)-entrapped liposomes (6×10^7), protoplasts (2.4×10^6), PEG (1%), mannitol (0.6 *M*) and phosphate buffer (50 m*M*) at *p*H 7.0. Neither $CaCl_2$, nor nutrient medium was included in the fusion assay[7]. Control in experiments related to the determination of liposome-protoplast fusion index (hereafter referred as fusion index) lacked PEG in the fusion assay. Fusion index denotes per cent fluorescing protoplasts among the number of viable protoplasts.

Plasmamembrane-enriched fraction (*PMEF*)—PMEF was prepared from different protoplast types essentially according to Galbraith and Northcote[22] with modifications to inhibit phospholipase-D and phosphatidicacid phosphatase activity[17].

Lipid analysis—Samples were fixed in 5 vol of boiling iso-propyl alcohol (g/v for callus, protoplasts and leaf; v/v for a dense PMEF) for 5 min prior to lipid extraction. Total lipids were extracted according to Bligh and Dyer[23] and the extract was washed with 0.2 vol 0.9% NaCl. The solvent phases were allowed to separate and the lower (chloroform) phase containing the total lipid was evaporated under reduced temperature and pressure. The concentrated lipid was stored under N_2 until required.

Lipid analysis was carried out as described earlier[17] and involved silica gel column chromatography and TLC. The methanol (galactolipid) fraction from column chromatography was concentrated and subjected to TLC on silica gel-H using chloroform:methanol:acetic acid:water (170:30:20:7) and the methanol (phospholipid) fraction was subjected to TLC on oxalic acid impregnated silica gel-H (0.04:1) with chloroform:methanol:water (65:25:4). The spots were visualized by I_2 vapour and their identities established. Determination of phospholipids and galactolipids was accomplished through lipid phosphorous and lipid galactose, respectively[24,25]. Protein was estimated according to Lowry *et al*.[26].

Data presentation—Protoplasts (300) were counted in each of the samples by taking out three aliquots. Protoplast counts were scaled down to 100 for expressing viability (%) and fusion index (%). Mean value and SD for protoplast counts were rounded off to whole number. Counting of fluorescing and non-fluorescing protoplasts was carried out for the determination of viability and fusion index. Protoplast viability (%) was included in calculating fusion index (%) and expressed as the number of fluorescing protoplasts among viable protoplasts. This was accomplished by independently determining the viability and fusion index of protoplasts for each sample under fusion assay conditions.

Results and Discussion

Objective of the present study was to work out a liposome-protoplast fusion system focusing on the following aspects– (a) optimization of conditions for liposome-protoplast fusion; (b) evaluation of plating efficiency of post-fusion protoplasts in relation to possible impairment or alteration of the functional state of protoplasts due to modification of plasmamembrane phospholipid-composition consequent to liposome-protoplast fusion and (c) evaluation of enrichment in the constituent phospholipid of liposomes in the plasmamembrane of post-fusion protoplasts. Experiments were aimed at working out a model system for specific modification of plasmamembrane phospholipid-composition of protoplasts via liposome fusion in the Solanaceae member. Whitish protoplasts were the choice over other protoplast types for optimization of parameters that influence liposome-protoplast fusion due to– (a) easy dispersal of cells and the consequent high protoplast-yield from this callus and (b) high degree of homogeneity of the callus.

Effect of FDA and PEG on the plating efficiency of protoplasts

In a set of preparative experiments, evaluation of the effect of specific components of the fusion assay, i.e. fluoroprobe and fusogen, was carried out on the functional state of protoplasts *in vitro*. In contrast to viability determination based on FDA-fluorescence which only reflects plasmamembrane integrity, plating efficiency of protoplasts reveal their functional state *in vitro*[5,7]. FDA (0.01%) and PEG (1%) were supplemented to the protoplast incubation-medium as specified prior to plating (Table 1). FDA or PEG did not affect plating efficiency of protoplasts when they were provided in the protoplast suspension either individually or in combination. Also protoplast-plating efficiency was not significantly affected by the neutral *p*H in the fusion assay.

Parameters that influence liposome-protoplast fusion

PEG-concentration and incubation duration—High molecular weight PEG as a fusogen *in vitro* is a

Table 1—Effect of FDA and PEG on plating efficiency of *D. innoxia* protoplasts
[Values are mean ± SD of 3 replicates]

Protoplast incubation condition	FDA	PEG	Plating efficiency (%)
Not fusion assay[a]	–	–	19.5 ± 0.98
Fusion assay[b]	–	–	18.8 ± 0.95
Fusion assay	+	–	18.6 ± 0.96
Fusion assay	–	+	18.3 ± 0.99
Fusion assay	+	+	18.4 ± 1.03

Protoplasts were incubated along with fusion-assay constituents prior to plating. Plating efficiency was determined after 23 days of plating; [a]cultured in nutrient medium at *p*H 5.6 without subjecting to either fusion-assay constituents or incubation conditions of the fusion assay; [b]subjected to *p*H 7.0 and 60 min incubation conditions of the fusion assay.

dehydrant by virtue of its binding water molecules with relatively high affinity leading to reduced availability of free-water molecules to ions and molecules in the incubation medium and also plasmamembrane molecules such as phospholipids, galactolipids, etc[27]. Stability of plasmamembrane of plant protoplasts that are in direct contact with incubation medium depends on the availability of sufficient free-water molecules[28]. Availability of free-water molecules contributes to optimal interaction with the amphiphilic lipids and other biomembrane molecules resulting in a functionally stable membrane organization[27,28]. Thus, PEG is associated with destabilization of biomembrane in a concentration dependent manner.

In the present study, in order to find out the effect of PEG concentration on the structural integrity of protoplasts, PEG at various concentrations, i.e. 0.5, 1, 1.5, 2 and 2.5%, was included in the protoplast incubation-medium. Subsequent light microscopic evaluation revealed that the protoplasts were deformed at PEG concentrations beyond 1% as compared to the control (Fig. 1C,D). Accordingly, in subsequent experiments PEG at 1% was employed in the fusion assay.

Protoplasts that are delivered with liposome-entrapped substances by employing PEG need to be free from any damaging effect of the fusogen on the functional state of post-fusion protoplasts, especially for subsequent growth and development of the protoplasts *in vitro*[7,8]. In this context, incubation duration in PEG might be critical in protecting the functional state of protoplasts under fusion assay conditions. Accordingly, effect of PEG-incubation duration on the plating efficiency of protoplasts was

Table 2—Effect of PEG-incubation duration on plating efficiency of protoplasts of *D. innoxia*
[Values are mean ± SD of 3 replicates]

Incubation duration (min)	Medium lacking PEG	Medium with PEG
0	18.6 ± 0.93	–
30	19.1 ± 0.94	18.4 ± 1.08
60	18.5 ± 0.98	17.6 ± 0.79
90	18.4 ± 1.06	14.9 ± 0.69
120	18.3 ± 0.97	6.9 ± 0.35

Protoplasts were incubated in PEG (1%) under fusion-assay conditions prior to plating. Plating efficiency was determined after 23 days of plating.

worked out by incubating protoplasts in the absence of liposomes for various durations in PEG and subsequently culturing them. Observations showed that the plating efficiency of protoplasts was significantly affected when the duration of incubation in PEG was >60 min (Table 2). Plating efficiency was decreased by ca. 24 and 65% when protoplasts were plated subsequent to PEG incubation for 90 and 120 min, respectively. Since PEG-incubation duration needed to be kept at the optimal level in relation to plating efficiency of the protoplasts, kinetics of delivery of liposome-entrapped FDA into protoplasts was not carried out. Retention of functional state of protoplasts under conditions of fusion assay was of prime importance in the present study. Accordingly, in the fusion assay employed in subsequent experiments PEG concentration and incubation duration were kept at 1% and 60 min, respectively.

Liposome composition

Liposomes of different composition have been routinely used for delivery of entrapped substances into animal cells and plant protoplasts[7,20,29-32]. Delivery of liposome-entrapped substances by phagocytosis-mediated uptake of intact liposomes into cell interior and subsequent destabilization of the liposomes in the cytosol have been demonstrated by employing acidic pH-sensitive liposomes[31]. Lectin-incorporated liposomes have been employed to increase fusion frequency of liposomes with protoplasts[30]. In the present study, liposomes differing in lipid composition and related surface charge were employed in the fusion assay in order to evaluate their suitability for the evaluation of incorporation of liposome phospholipid in the plasmamembrane of post-fusion protoplasts (Table 3).

Results showed that the neutral liposomes prepared solely from PC performed well in the fusion assay

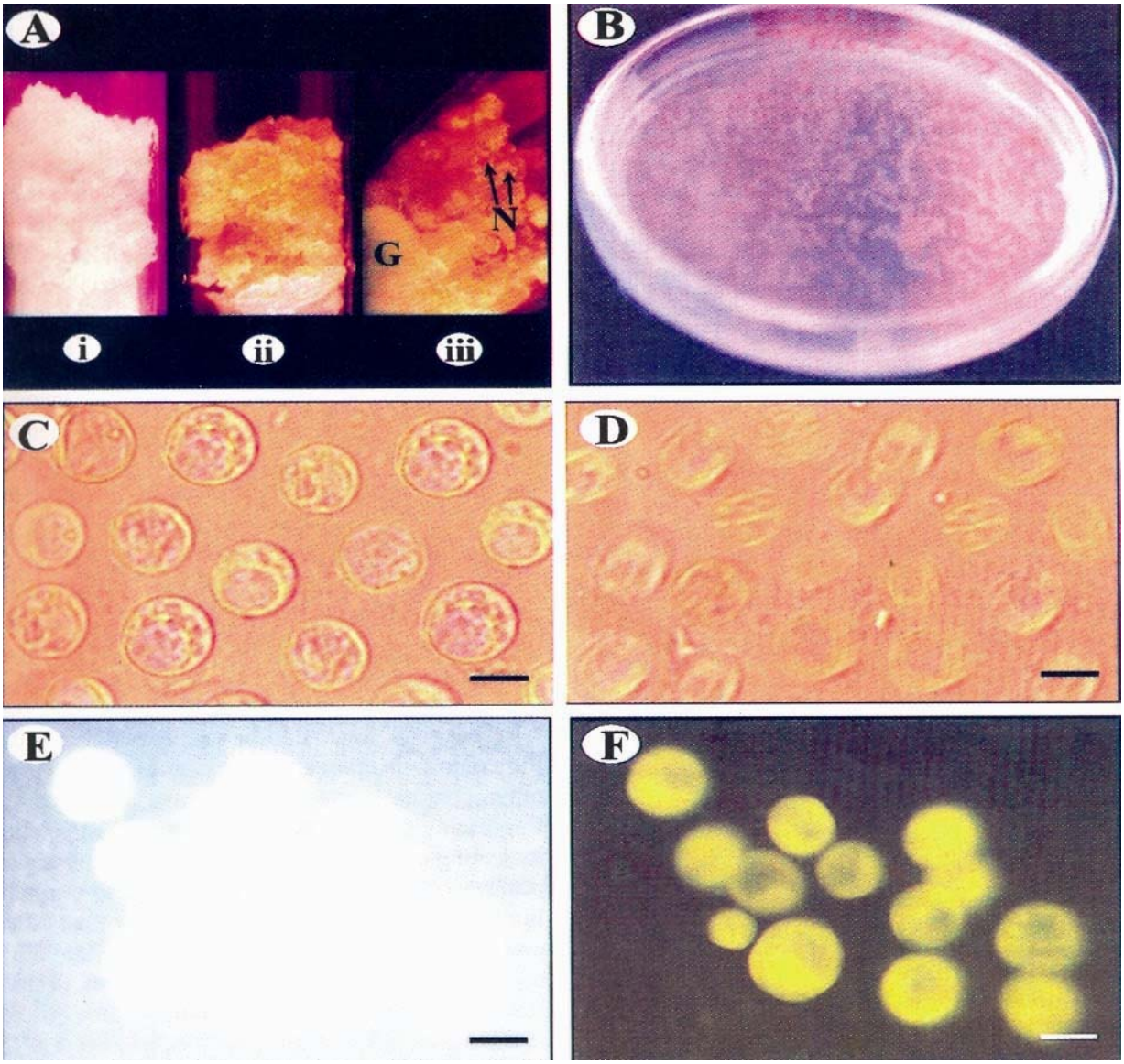

Fig. 1—Components of liposome-protoplast fusion system of *D. innoxia* [A) callus cultures– i) whitish; ii) green; iii) shoot differentiating with green region (G) and nodules (N); (B) microcalli formed in protoplast cultures after 23 days of plating; protoplasts incubated in 1% (C) and 1.5% (D) PEG for 60 min under fusion assay conditions; whitish protoplasts subsequent to incubation in fusion assay viewed in phase-contrast (E) and fluorescence (F) microscopy field; Bar = 20 µm].

resulting in a relatively high fusion-index. The order of performance of different type of liposomes based on fusion index was as follows– PC>PC+SS>PC+SA>PC+SS+SA>PC+DP>PC+SS+DP. Sterol inclusion along with phospholipid(s) has been shown to stabilize liposomes both *in vivo* and *in vitro* especially in systems involving serum[20,29]. Sterol-mediated stability of liposomes was attributed to protection of liposomes from phospholipases attack due to relatively tight packaging of the membrane molecules in sterol-incorporated liposomes. In the present study, based on the performance of different liposomes in the fusion assay, PC-liposomes were found ideal for subsequent experiments due to– (a) relatively high fusion index observed and (b) fulfilling the requirement of a relatively simple evaluation of enrichment in the PC-content in the plasmamembrane of protoplasts subsequent to liposome-protoplast fusion. Ongoing studies in our laboratory are focused on metabolic conditioning of protoplasts by

incorporating specific phospholipid(s) in the plasmamembrane by employing liposome-protoplast fusion system. Though the change in membrane properties brought about by liposomes will be transitory such a change would be critical for the protoplasts for their initial establishment in culture. Through supplementation of appropriate membrane-lipid precursors in the culture medium, further growth and development of protoplasts *in vitro* could be ensured. Thus employing the liposome-protoplast fusion system is aimed to achieve optimal growth in the early stage of protoplast cultures. Further, this methodology would augment the efforts made on extracellular parameters such as improvement of culture medium and culture environment[9,10].

Liposome-protoplast ratio

In this set of experiments, a constant protoplast-density (2.4×10^6 whitish protoplasts) equivalent to 1 unit of protoplasts was employed in the fusion assay wherein liposome density alone was varied (Fig. 2).

Table 3—Effect of liposome composition and surface charge on the fusion index of protoplasts of *D. innoxia*
[Values are mean ± SD of 3 replicates]

Liposome composition	Fusion index (%)
Phosphatidylcholine (neutral)	81 ± 5
Phosphatidylcholine + stigmasterol (neutral)	74 ± 5
Phosphatidylcholine + sterylamine (positive)	67 ± 4
Phosphatidylcholine + stigmasterol + sterylamine (positive)	64 ± 4
Phosphatidylcholine + dicetyl phosphate (negative)	58 ± 3
Phosphatidylcholine + stigmasterol + dicetyl phosphate (negative)	33 ± 3

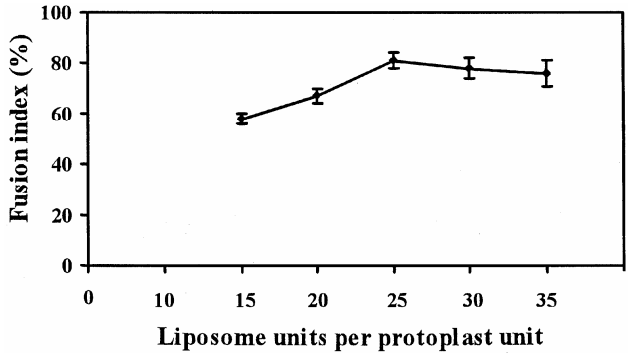

Fig. 2—Effect of liposome:protoplast ratio on the fusion index of protoplasts of *D. innoxia* [Fusion index as per cent fluorescing protoplasts among viable protoplasts was determined. 1 unit of protoplasts was 2.4×10^6 in the fusion assay. PC-liposomes were employed in the fusion assay].

Results showed that there was no detectable level of liposome-protoplast fusion based on the fusion index of protoplasts up to liposome:protoplast ratio of 10:1. Fusion index was detectable only when liposome:protoplast ratio was 15:1 and it showed linear increase up to 25:1 beyond which there was a plateau. At 25:1 liposome:protoplast ratio, fusion index was found to be the maximum at ca. 81%. These observations indicate the existence of fusogenic and non-fusogenic protoplasts in the sample together with differences in the occurrence of available fusion sites in the plasmamembrane of fusogenic protoplasts under the specified experimental conditions[7]. It has been proposed that there are specific membrane contact-sites and fusion sites in the plasmamembrane and hence it is possible to achieve saturation of the fusion sites in a liposome-protoplast fusion system[1,32].

Fusion index of different protoplast types

In this set of experiments fusion index of the different protoplast types was determined. PEG was omitted in the control in order to exclude the possibility of liposome instability and consequent leakage of FDA into the assay mixture contributing to false positives for fusion index. It was observed that the control in this set of experiments as well as in other fusion-assay experiments showed 0% fluorescing protoplasts under the specified conditions indicating optimal stability of liposomes and also non-occurrence of spontaneous uptake of FDA-entrapped liposomes by the protoplasts. Viability of the different protoplast types was in the range of ca. 90-96% and the fusion index was based on this value (Table 4). Results showed that there was significant difference in the fusion index of different protoplast types. There was apparently an inverse relationship between the degree of differentiation of the protoplast types and the fusion index.

It is known that the mechanism of delivery of liposome-entrapped substances in the cytosol differs

Table 4—Viability and fusion index of different protoplast types of *D. innoxia*
[Values are mean ± SD of 3 replicates]

Protoplasts	Viability (%)	Fusion index (%)
Whitish	96 ± 2	81 ± 5
Green	94 ± 2	62 ± 4
Green region; shoot differentiating callus	93 ± 2	59 ± 3
Nodules; shoot differentiating callus	90 ± 4	43 ± 3
Leaf	95 ± 4	34 ± 2

depending on the type of liposomes employed[1,31]. Unilamellar (UL) liposomes deliver entrapped substances either by fusion with the plasmamembrane or by phagocytosis followed by destabilization in the cytosol[1,20]. ML liposomes on the contrary are known to involve a complex mechanism in the delivery of entrapped substances. In the case of membrane-fusion route involving ML liposomes, their outermost lamella gets stripped off subsequent to fusion with the plasmamembrane and the stripped-off liposomes that reach the cytosol subsequently get destabilized and release their contents[20]. In this regard acidic-*p*H sensitive liposomes, which get destabilized at weak acidic-*p*H of ca. 6.0, have been widely employed due to their rapid destabilization in the cytosol where the required *p*H exists *in situ*[31]. It has also been shown that PC-liposomes undergo rapid destabilization in the cytosol in ca. 2 min[29]. Observations of the present study point to the possibility that the delivery of liposome-entrapped FDA in the cytosol of protoplasts was total due to the short-lived nature of PC-liposomes in the cytosol irrespective of whether the liposome preparation of the present study consisted of homogeneous population of UL liposomes or heterogeneous population consisting of both UL and ML liposomes. Characterization of the UL and ML nature of the employed liposomes was beyond the scope of the present study.

In the present study, the protoplast types showed varying levels of fusion index, though not 100% even in anyone of the protoplast types, indicting the occurrence of both fusogenic and non-fusogenic protoplasts in the different protoplast types (Fig. 1E,F; Table 4). Depending on the presence and availability of fusion sites in the plasmamembrane fusion index has been suggested to vary[7,31]. Evaluation of fusion index, especially in systems comparable to that of the present study in contrast to simple liposome-liposome fusion systems, is found to be difficult due to the complexity of biomembrane phenomenon that occurs in the microdomains of membrane[27,32].

Plating efficiency of different post-fusion protoplasts

This set of experiments was carried out to find out the growth ability of protoplasts *in vitro* subsequent to the fusion of PC-liposomes with protoplasts. Predicted incorporation of liposome-PC in the plasmamembrane might eventually lead to increase in the relative level of PC in relation to other membrane lipids of the plasmamembrane besides an absolute increase in PC content in the post-fusion protoplasts. It is known that the stoichiometry of different membrane-lipids in the biomembrane is functionally significant in relation to the activity of membrane-bound sub-systems such as enzymes, receptors, carrier proteins, etc[33]. In the present study, it was of interest to see what functional significance would be due to the modification of plasmamembrane PC on the growth ability of post-fusion protoplasts *in vitro*. Accordingly, whitish- and green- protoplasts that were fused with PC-liposomes in the fusion assay were employed for determining their post-fusion plating efficiency.

Observations showed that there was significant increase in the plating efficiency of the protoplasts subsequent to fusion of PC-liposomes with the protoplast types as compared to their respective controls (Table 5). There was ca. 41 and 21% increase in the post-fusion plating efficiency subsequent to fusion of PC-liposomes with whitish- and green-protoplasts, respectively. Plating efficiency of the pre- and post-fusion protoplast cultures observed in the present study is comparable to that of the carrot and tobacco cultures[5,7]. Even though a report on the *in vitro* manipulation of membrane lipids by supplementation of membrane-lipid precursors in callus cultures of *D. innoxia* associated with change in the differentiation responses is available, there is no report on the growth ability of protoplasts due to modification of plasmamembrane lipid-composition via liposome-protoplast fusion[16]. Thus, the observations of the present study provide evidence for the first time on the increased plating efficiency of protoplasts due to modified plasmamembrane lipid-composition as a result of the fusion of PC-liposomes with protoplasts.

Enrichment of PC in post-fusion protoplasts subsequent to fusion of PC-liposomes

Observations on the post-fusion plating efficiency pointed to the possible modification of

Table 5—Plating efficiency of post-fusion protoplasts of *D. innoxia*
[Values are mean ± SD of 3 replicates]

Protoplasts	Plating efficiency (%)	
	Medium lacking PEG	Medium with PEG
Whitish	18.9 ± 1.17	26.7 ± 1.26
Green	15.0 ± 0.87	18.1 ± 0.83

Table 6—Change in the plasmamembrane polar-lipids due to fusion of PC-liposomes with protoplasts of *D. innoxia*
[Values are mean ± SD of 3 replicates]

Protoplast PMEF	Polar-lipid content (nmole mg^{-1} protein)		Phospholipids (%)					
	Phospholipids	Galactolipids	PC	PE	PI	PS	PG	PA
Whitish; pre-fusion	840 ± 19	36 ± 1	45	26	17	6	2	4
Whitish; post-fusion	896 ± 22	39 ± 1	49	23	16	6	2	4
Green; pre-fusion	900 ± 22	53 ± 2	44	29	14	7	3	3
Green; post-fusion	945 ± 21	58 ± 2	47	27	13	7	3	3

Table 7—Change in the plasmamembrane PC- and PE-content due to fusion of PC- liposomes with whitish- and green- protoplasts of *D. innoxia*
[Values are mean ± SD of 3 replicates]

Protoplast PMEF	Phospholipids (nmole mg^{-1} protein)	
	PC	PE
Whitish; pre-fusion	378 ± 9	218 ± 5
Whitish; post-fusion	437 ± 11	212 ± 5
Green; pre-fusion	396 ± 11	261 ± 7
Green; post-fusion	445 ± 9	255 ± 7

plasmamembrane PC that resulted in growth stimulation of the protoplasts *in vitro* (Table 5). In order to substantiate as to whether the delivery of liposome-entrapped FDA into protoplasts was associated with liposome-protoplast fusion in this system, plasmamembrane phospholipid-composition especially in relation to the content of PC– the constituent phospholipid of the employed liposomes, was determined. Whitish- and green-protoplasts were employed in this set of experiments as these protoplast types showed relatively higher fusion index (Table 4).

Results showed that there was no significant difference in the total content of phospholipids and galactolipids in the PMEF of the whitish- and green-protoplasts (Table 6). Qualitatively, PMEF of both the protoplast types had identical membrane-lipid composition consisting of PC, phosphatidyl-ethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidicacid (PA) among phospholipids and MGDG, DGDG and SL among galactolipids. Individual galactolipids of different PMEF were present as minor constituents amounting together to only ca. 6% of the total phospholipids + galactolipids content (data not shown). PA, a lipolytic product of other phospholipids due to phospholipase-D activity, was present in very low levels at ca. 4% of total phospholipids in the PMEF of whitish as well as green protoplasts, indicating the absence of lipolytic degradation of phospholipids during the isolation of PMEF[20].

Subsequently, evaluation of PC content of PMEF was carried out for assessing the post-fusion change in this constituent phospholipid. PE content was evaluated along with that of PC in the PMEF with the aim of keeping PE content as a positive control due to– (a) the predominance of PE next only to PC in the PMEF of the protoplasts and (b) its absence in the employed liposomes (Table 6). Results showed that there was ca. 16 and 12% increase in the PC-content of post-fusion PMEF of the whitish- and green-protoplasts, respectively. However, there was no significant change in PE content of the PMEF of either the whitish- or green-protoplasts. PC enrichment as a function of total phospholipid-content was ca. 7 and 5% in the PMEF of whitish- and green-protoplasts, respectively (Tables 6, 7). Similar results were obtained when liposomes lacking FDA were employed (data not shown). Results of the present study indicate that manipulation of plasmamembrane phospholipids via liposome-protoplast fusion offers an attractive tool to metabolically condition the protoplasts for achieving desired performance of protoplasts *in vitro*[21,29].

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