Encapsulation of Bacteria-Derived Auxin, Cytokinin and Gibberellin and its Application in the Micropropagation of Coconut (Cocos nucifera L. var Makapuno)

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Abstract

Bacteria-derived crude auxin, cytokinin and gibberellin were isolated from plant gowth-promoting bacteria containing 826.43 ± 3.3 mg/g IAA equivalence, 489.66 ± 1.0 mg/g Kinetin equivalence and 588.90 ± 6.3 mg/g Gibberellic acid equivalence respectively. The isolated plant growth regulators (PGRs) were also successfully encapsulated, using phospholipid and β-sitosterol with encapsulation efficiency of $82.6 \pm 0.50\%$, $84.6\% \pm 0.01\%$ and $66.75 \pm 0.62\%$ for indole-3-acetic acid (IAA)-loaded, cytokinin (Ck)-loaded and gibberellin (GA)-loaded liposomes respectively.

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Improved solubility of the PGR-loaded liposomes in aqueous solution was also observed. The PGR-loaded liposomes showed controlled-released behavior of the active component at pH 5.0 and 7.0. Higher stability of the encapsulated PGRs was observed at 0 - 4°C for 3 months.

The effect of the PGR-loaded liposomes on the callus initiation of makapuno (*Cocos nucifera* L. var Makapuno) was also evaluated. Out of 15 treatments used in the study, 8 gave an initial callus formation with high frequency (> 75%). Considering the color, shape and frequency of the formation of initial callus, supplementation of 100% IAA-loaded liposomes on the medium gave the highest acceptable initial callus formation for the makapuno (*Cocos nucifera* L. var Makapuno). Initial callus formation was observed at 8 days following culture in contrast to the unencapsulated IAA which took about 20 days before initial callus formation.

Uptake of the IAA-loaded liposomes with PbS quantum dots (QDs) was observed even after 5 minutes only following culture. No entry pattern based on fluorescence in initial callus observed in the uptake of the IAA-loaded liposomes-PbSQD hybrid.

Keywords: Encapsulation; tissue culture; coconut; plant growth regulator.

1. Introduction

Phytohormones are a set of compounds that gives certain response to the plants mostly for its overall growth and development. Hormones are released to all cells in small concentrations, but only specific cells that have the special receptors for each hormone will react leading to the stimulation of its specific functions. There are five classical hormones namely auxin, cytokinin, abscisic acid, gibberellin and ethylene.

Auxin is a group of compounds that primarily promotes cell elongation and differentiation in plants. Woodward and Bartel [1] reported that auxin also promotes cell division and elongation, differentiation, tropism, apical dominance, senescence, abscission, and flowering. Cytokinin (Ck) is the phytohormone generally attributed for cell division, though it is also responsible for chlorophyll maturation, inhibiting leaf senescence, and nutrient movement. Gibberellins (GA) are a group of diterpene phytohormone synthesized mostly via mevalonic acid (MVA) pathway. It is first observed in bakanae disease; an abnormal rice growth cause by the fungus *Gibberella fujikoroi*. The key feature of gibberellins is in amylase production during seed germination. Plant growth regulators are reported to be produced by bacteria also. They were already isolated and characterized by Difuntorum-Tambalo and his colleagues [2], Ereful and his colleagues [3] and Fernando and his colleagues [4] from bacteria.

In different areas of agriculture, application of exogenous plant growth regulator (PGR) plays a very important role in crop propagation and greater crop yield production. However, several problems have been associated with external application of these PGRs such as solubility, volatility for foliar application and cellular uptake to the explants for tissue culture and/or cell suspension culture. These problems could possibly be overcome by encapsulating the plant growth regulators.

Encapsulation is a technique in coating several substances within another material at nano or micro scale sizes. It gives protection of the sensitive bioactive compounds from harmful and unfavorable environmental conditions, increase solubility and eliminate incompatibilities with other substances in a given mixture. Liposomes are also used to encapsulate highly bioactive enzymes in dairy products [5]. Nanoencapsulation have been extensively studied in the past few years as a technique in incorporating substances in particulate carriers in the pharmaceutical and medical fields, because of their high potentials as drug delivery systems attributed to its controlled- and sustained-release properties, subcellular size, and biocompatibility with tissue, cells and any other components in a living system matrix [6].

One of the areas in agriculture where PGRs are very useful is in the micropropagation of coconut. Coconut is considered as one of the high-valued crop in the Philippines. An estimated 25 million Filipinos rely upon the coconut industry where 3.5 million of whom are coconut farmers [7]. In 2010, the Philippine coconut industry faced a problem due to the coconut scale insect (*Aspidiotus rigidus* R.) infestation leading to a damaging effect on the livelihood of many Filipino. With the decrease in the production of coconut due to several factors, there is a need to maximize the micropropagation of coconut especially those variety with collapsed embryo such as the makapuno which is an important raw material in the production of specialty food products.

This study aimed to encapsulate bacteria-derived plant growth regulators to evaluate controlled release, improved solubility and bioactivity. The study further assessed the fate of the encapsulated PGRs in tissue cultured plants. The projected output of the study will be beneficial to plant growers especially those dealing more on tissue culture.

2. Materials and Methods

2.1 Chemicals and Materials

All chemicals and reagents used were analytical grade and were purchased either at Sigma, Univar and/or Scharlau. Food grade soy lecithin was purchased from a local supplier. All bacterial isolates were provided by the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños.

2.2 Preparation of Growth Medium

Auxin-producing bacteria (PGPB 1) were incubated in minimal salt edium (MSM) broth [8]. In a 1000-ml beaker KH_2PO_4, Na_2HPO_4, and $MgSO_4.7H_2O$ (Scharlau) were dissolved in distilled water then added with a solution of trace elements. The trace elements solution contains $CaCl_2.2H_2O$, $FeSO_4.7H_2O$, $MnSO_4.H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, H_3BO_3, $CuCl_2.6H_2O$, $Na_2MoO_4.2H_2O$ and conc. H_2SO_4. The pH was adjusted to 7.0 and autoclaved for 15 minutes. In a separate container, glucose, L-tryptophan (partially dissolved in NaOH) and yeast extract were dissolved in sterile distilled water. The two solutions were combined, mixed thoroughly and maintained at 30°C under dark condition. Gibberellin-producing bacteria (PGPB 4) were incubated in King's Medium B (KMB) broth [9]. Peptone, $MgCl_2$, K_2SO_4 were dissolved in distilled water and added with glycerol, mixed thoroughly and autoclaved for 15 minutes. Cytokinin-producing bacteria (PGPB 2) were incubated in an

amended KMB [3]. The standard KMB were added with sucrose, KNO_3, adenine and isopentyl alcohol then mixed thoroughly and autoclaved for 15 minutes.

2.3 Production and Extraction of Auxin, Cytokinin and Gibberellin

The production and extraction of auxin, cytokinin and gibberellin from bacterial isolates followed the procedure described by Difuntorum-Tambalo and his colleagues [2], Ereful and his colleagues [3] and Fernando and his colleagues [4], respectively. IAA-producing bacteria were incubated in MSM for 14 days with shaking under room temperature. After the incubation period, the medium was centrifuged at 10 000 rpm for 30 minutes, then decanted. The pH of the supernatant was adjusted to 8.1 with Na_2CO_3 and partitioned with ethyl acetate. The aqueous portion was separated and the pH was adjusted to 2.8 with H_3PO_4 and partitioned with ethyl acetate. The ethyl acetate portions were then pooled and reduced to residue under vacuum. Cytokinin-producing bacteria were incubated in amended KMB for 48 hours with shaking under room temperature. It was centrifuged at 10 000 rpm for 30 minutes then decanted. The supernatant was partitioned with ethyl acetate and butanol. The organic portions were reduced to residue using N_2 gas. Gibberellin-producing bacteria were incubated in KMB for 14 days with shaking under room temperature. It was then centrifuged at 10 000 rpm for 30 minutes and decanted. The supernatant was freeze-dried to reduce its volume (1/10). The pH of the reduced supernatant was adjusted to 2.5 with 1.0N HCl and partitioned with ethyl acetate. The ethyl acetate portion was then partitioned with 5% $NaHCO_3$. The aqueous portion was partitioned with ethyl acetate. The ethyl acetate portion was reduced to residue under vacuum.

The presence of plant growth regulators in the residues was evaluated using colorimetric and bio-assay; Salkowski test for Auxin, radish cotyledon expansion assay for cytokinin and dwarf rice assay for gibberellin. The amount of active compound of the extracted PGRs were quantitatively determined using Salkowski's test while for cytokinin and gibberellin it was determined spectrophotometrically at 269 and 254 nm respectively using a UV-Visible spectrophotometer (Shimadzu UV-1800 Japan). Results were expressed as IAA, kinetin and gibberellic acid equivalence for the crude auxin, cytokinin and gibberellin respectively.

2.4 Extraction of Phospholipid

The extraction of phospholipids from soya bean lecithin follows the procedure described by Wu and Wang [10] and Jangle and his colleagues [11], with some modifications. Crude lecithin was deoiled with acetone. Phospholopids were extracted using ethanol and isopropyl alcohol (4:3). The alcohol soluble fraction was dried using a rotary evaporator. The residue was then washed with cold acetone and dried using N_2 gas and used for the preparation of liposomes.

2.5 Preparation of PGR-loaded liposomes

Liposomal PGRs were prepared using thin film hydration method or Bangham method [12][13] with some modifications. Phospholipid, β-sitosterol and individual PGRs; indole-3-acetic, cytokinin, and gibberellin (3:1:1, 2:0.67:1, 5:1.67:1, w/w respectively) were dissolved in ethanol at 50°C. The solution was transferred to a flask and dried in a rotary evaporator to form thin lipid film and was flushed with N_2 gas. The film was re-suspended

in phosphate buffer (0.05 M and pH 7.2) to produce multilamellar liposomal vesicles. It was then homogenized and sonicated for 60 minutes at 25°C and was freeze-dried for 3 days.

2.6 Determination of Encapsulation Efficiency

The percentage of PGR encapsulated was determined. The prepared liposomes were subjected to lysis with absolute alcohol and were sonicated for 10 minutes. The concentration of IAA in the absolute alcohol was determined using Salkowski's test while for cytokinin and gibberellin it was determined spectrophotometrically at 269 and 254 nm respectively using a UV-Visible spectrophotometer (Shimadzu UV-1800 Japan) in triplicate. The encapsulation efficiency expressed as entrapment percentage was calculated through the following relationship as described by Gulati and his colleagues [14]:

Encapsulation Efficiency (%) = [(Total PGR – Free PGR)/Total PGR] x 100

2.7 Characterization of PGR-loaded Liposomes

Particle Size Distribution. Liposomes were also evaluated for size and size distribution using Zetasizer (Malvern Ver. 7.02: MAL1087943). Sample of liposomes were dissolved in distilled water and were analyzed using the said instrument at 25° for 15 minutes.

Determination of Solubility. Equal amount (10 mg) of the encapsulated samples, unencapsulated crude plant growth regulators and standard (commercially available) plant hormones were dissolved in 3 mL distilled water and shake for 1 hr. Ensuring that no particles were included, an aliquot (50 μL) of the samples were diluted in 10 mL distilled water. Solubility of plant growth regulators were analyzed spectrophotometrically.

Storage Stability study. The freeze dried PGR-loaded liposomes were separated into three groups kept at 0°C, 4°C and 27°C for three months. The samples subjected to stability tests were analyzed for PGRs equivalent using UV-Vis spectrophotometry after the storage period of three months.

In vitro release studies. Controlled-release from PGR-loaded liposomes was studied using a dialysis method by Panwar and his colleagues [15] with some modifications. Dialysis bags were soaked before use in distilled water at room temperature for 12 hours to remove the preservative, and rinsed thoroughly in distilled water. In vitro release of PGRs from liposomes was conducted in a dialysis sac (Sigma, 12,000 MW cut off) with 150 mL of phosphate-buffered saline (PBS; pH 5.6) containing 7% (V/V) propylene glycol and 25% (V/V) methanol at 37°C. Control sacs were also prepared containing free PGRs and encapsulated PGRs. Liposomal concentrate (equivalent to 2mg PGRs) was dispersed in 1 mL of bicarbonate buffer (pH 9) and placed in a dialysis bag. Control bags were prepared and tested along with the liposomal dispersions. Each control bag contained 2 mg of PGRs. Two ends of the dialysis sac were tightly bound with threads and hung inside a conical flask reassuring that the portion of the dialysis sac with the formulation was dipped into the buffer solution. The flask was kept on a magnetic stirrer at 37°C with a thermostatic control. Samples were collected every half an hour over a period of five hours and assayed spectrophotometrically for PGR content at 269 and 254 nm for cytokinin and gibberellin respectively and Salkowski's test for IAA.

2.8 Effect of Encapsulated PGRs on Makapuno Tissue Culture

Makapuno (*Cocos nucifera* L.), of VSU, Makapuno 2 (VMAc2; NSIC 2008 Co-28) cultivar, plumule was used. VMac 2 was harvested at the local plantation of the National Coconut Research Center at the Visayas State University. It was dehusked, opened and cylinder endosperm, near the "eye" of the coconut shell, was removed which contained the embryo. The cylinder embryos were sterilized using detergent and 20% NaOCl; using a laminar hood, the embryo was removed and placed in sterile double distilled water. Plumule was removed from the embryo, sliced into 6-8 parts then planted in modified MS medium containing the PGR-loaded liposomes in varying ratios. The cultures were observed and data were collected in a span of 1 month.

A total of 15 ratios, which serves as treatment, were used for the formulation of PGR-loaded liposomes. The ratio with the highest effectiveness was compared using the same tissue culture method with medium containing free PGRs, from bacteria; with liposome alone, and with water only. Color and shape were scored based on the description reported by Perez-Nuñez and his colleagues [16]. For the color, brown (1 - 1.9), light brown (2.0 – 2.9), beige (3.0 – 3.9), white (4.0 – 5.0) were used and for the shape; bulging (1.0 – 1.9), < 1 mm (2.0 – 2.9), intact (3.0 – 3.9) and presence of globular structure (4.0 – 5.0).

2.9 Uptake of IAA-loaded Liposome-PbS Quantum Dots Hybrid in Makapuno Initial Callus

Tracer study was conducted using PbS quantum dots (PbSQD) provided by Dr. Blessie A. Basilia of Industrial Technology Development Institute - Department of Science and Technology (ITDI-DOST), Taguig City, Metro Manila. The IAA-loaded liposome was coated to the quantum dots following the lipid film hydration method as described by Tahara and his colleagues [17] and Dubertret and his colleagues [18] with some modifications. The IAA-loaded liposome-PbSQD hybrids were supplemented to MS medium. Initial callus was incubated to the medium. Incorporation of the IAA-loaded liposome-PbSQD hybrid was assessed using an epifluorescent/light microscope (Zeiss Axioplan 2 with Olympus DP70 camera). Observations were taken after 5 minutes, 1 hour, 3 hours and 5 hours following culture.

2.10 Experimental Design

Formulation of encapsulated PGR mixture was carried out using Simplex Lattice Design (SLD), in 5 levels generating 15 test ratios which serve as treatment with ANOVA. Analyses of the main and interaction effects of the independent variables, or the treatments, on the monitored parameters were done using SAS Mixed Model procedure with $\alpha=0.05$ and contour plotting. Data were analyzed using Minitab 16 and contour and surface plots were generated. Analysis and prediction of the optimized component were also generated.

3. Results

The study focused mainly on the encapsulation and physicochemical characterization of bacteria-derived crude auxin, cytokinin and gibberellin only and its application on the micropropagation of coconut (*Cocos nucifera* L. var Makapunno) specifically on the callus initiation stage.

3.1 Crude Plant Growth Regulators from Bacteria

Crude plant growth regulators (PGR) were successfully isolated from plant-growth promoting bacteria. Crude auxin extract has a characteristic light brown with greenish shade color while crude cytokinin, from butanol portion, is white and crude gibberellin is dark brown. Crude auxin and cytokinin are solid while gibberellin is liquid. All residues have a pungent odor which is an evident characteristic of any bacterial remnants. The pooled crude auxin has 826.43 (±3.3) mg g-1 IAA equivalence while the crude cytokinin (Ck) extract has 489.66 (±1.0) mg g-1 Kinetin equivalence and the crude gibberellin (GA) has 588.90 (±6.3) mg g-1 Gibberellic acid equivalence.

3.2 Fabrication of PGR-loaded Liposomes

PGR-loaded liposomes were successfully produced using PC-enriched phospholipids and β-sitosterol in the following lipid-sterol-hormone ratio; IAA (3:1:1), Cytokinin (2:0.67:1), Gibberellic acid (5:1.67:1) following the lipid-film hydration method. The fabricated IAA-loaded liposomes were compact in an agglomerate form while the Ck-loaded liposomes were powder and the GA-loaded liposomes were flaky and some were forming agglomerates also. All PGR-loaded liposomes were hygroscopic which is commonly observed in lyophilized samples. The encapsulation efficiency of the PGR-loaded liposomes was presented in Table 1. IAA-loaded and cytokinin-loaded liposomes have more than 80% encapsulation efficiency while gibberellin-loaded liposome has 66.7 % (± 0.62) encapsulation efficiency.

Table 1: Encapsulation efficiency (%) of the PGR-loaded liposomes

Hormone	% Encapsulation Efficiency
IAA	82.60 ± 0.50
Ck	84.60 ± 0.01
GA	66.75 ± 0.62
Results presented are mean (n=3) ± standard deviation	

3.3 Particle Size and Distribution

The sizes of the encapsulated PGRs were determined using a Zetasizer. Result shows that two distinct sizes were detected (Table 2) at different peaks

IAA-loaded and GA-loaded liposomes gave bigger sizes at peak 1 while Ck-loaded gave less than 1000 nm size at Peak 1. All of the encapsulated PGRs gave less than 500 nm size at peak 2.

3.4 Solubility Test

Solubility test was conducted for the PGR-loaded liposomes in comparison with a standard PGR and free crude

PGR from bacteria. In the study, the standard plant growth regulators as well as the unencapsulated crude PGRs did not dissolve in water while the PGR-loaded liposomes dissolve in aqueous solution.

The solubility of the samples was 2.75 mg mL^{-1} ($\pm$ 0.07), 2.41 mg mL^{-1} ($\pm$ 0.03) and 4.32 mg ml-1 ($\pm$ 0.09) for IAA-loaded, Ck-loaded and GA-loaded liposomes respectively. The crude cytokinin extract however, has a solubility of 0.38 mg mL-1 ($\pm$ 0.01); solubility of cytokinin was increased in more or less 6 fold in the encapsulated form. Solubility of the standard PGRs and crude IAA and gibberellin extracts were not identified since it has undetectable absorbance.

Table 2: Size of the PGR liposomes observed at different region (peak)

Sample	Size (d.nm)	
	Peak 1	Peak 2
IAA-lnL	1322.50	303.00
Ck-lnL	891.75	170.80
GA-lnL	1212.00	179.90

3.5 In Vitro Release Study

Evaluation of in vitro release of the PGR-loaded liposomes was done by dialysis method. The in vitro release behavior was summarized in Figure 1.A for IAA-loaded liposome, Figure 1.B for Ck-loaded liposome and Figure 1.C for GA-loaded liposome.

Analyses were carried out at pH 5.0 and 7.0 simulating an extracellular and cytoplasmic pH condition respectively and were evaluated for a period of 24 hours (1440 minutes). Free crude PGR were also tested for comparison.

In the case of the IAA, for pH 5.0, maximum release (> 80%) of the free crude auxin extract was observed after 1 and a half hour. Considering the unencapsulated IAA was crude, with an active component of < 85%, this release was considered to be its optimum. The same effect was also observed at pH 7.0. Also, maximum PGR release was observed after 1 to 2 hours for the crude Ck and GA extract at both pH levels. Less than 100% were observed in all the unencapsulated PGR since it is in its crude form suggesting that any detection of PGR active components beyond this time can be attributed to the high concentration in the matrix and cannot be considered as the actual release.

3.6 Storage Stability

The stability of PGR-loaded liposome under different temperatures (27°C, 4°C and 0°C) were assessed in the presence of active component as shown in Figure 2.

There is no significant decrease on the amount of PGR in the samples stored at 4°C and 0°C ($P > 0.05$) for a span of 3 months but has significantly decreased for the samples stored at 27°C ($P < 0.05$). These observations are true to all the PGR-loaded liposomes (Table 3). The result also shows a higher stability of the liposomes under low temperature.
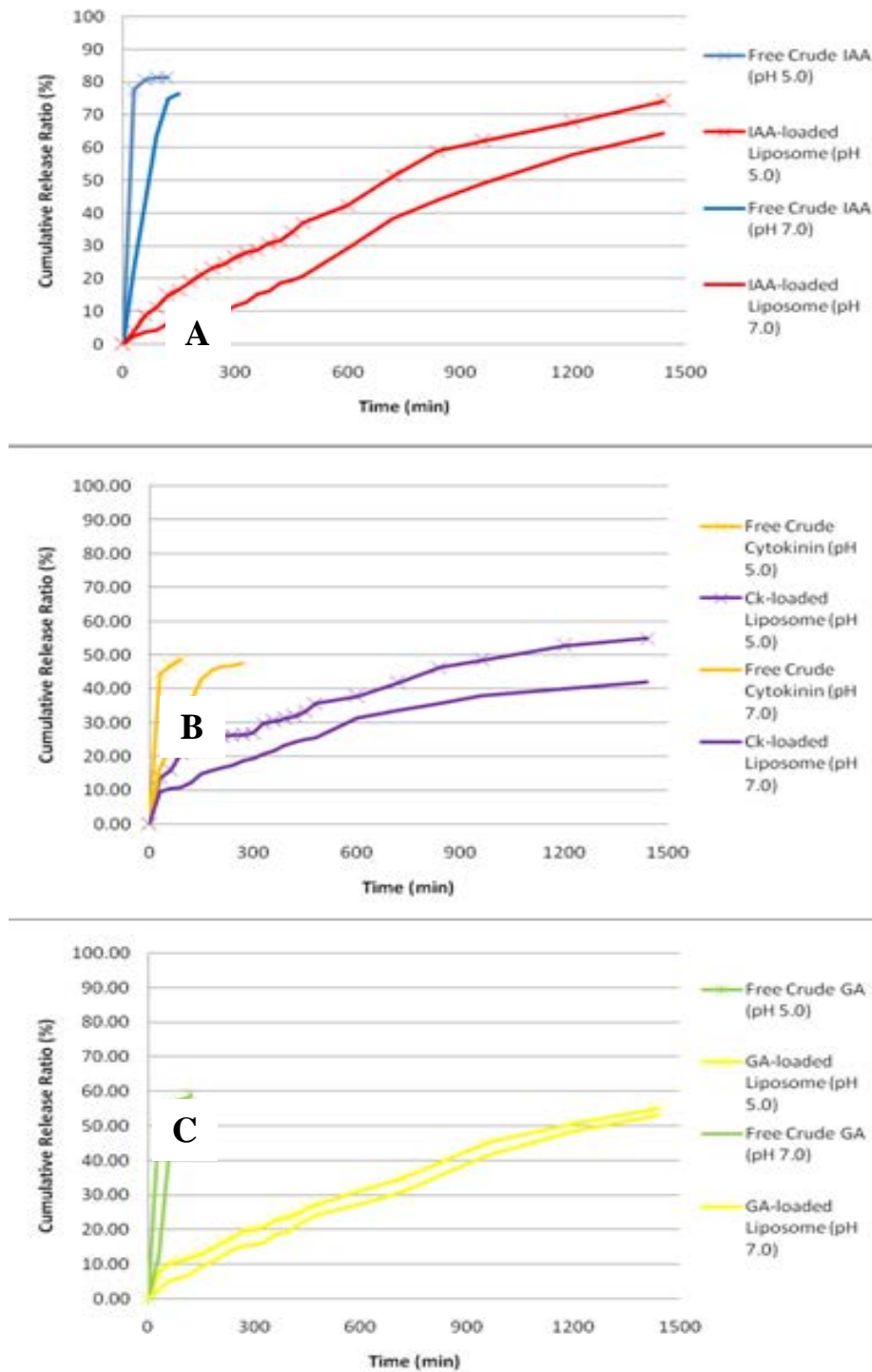
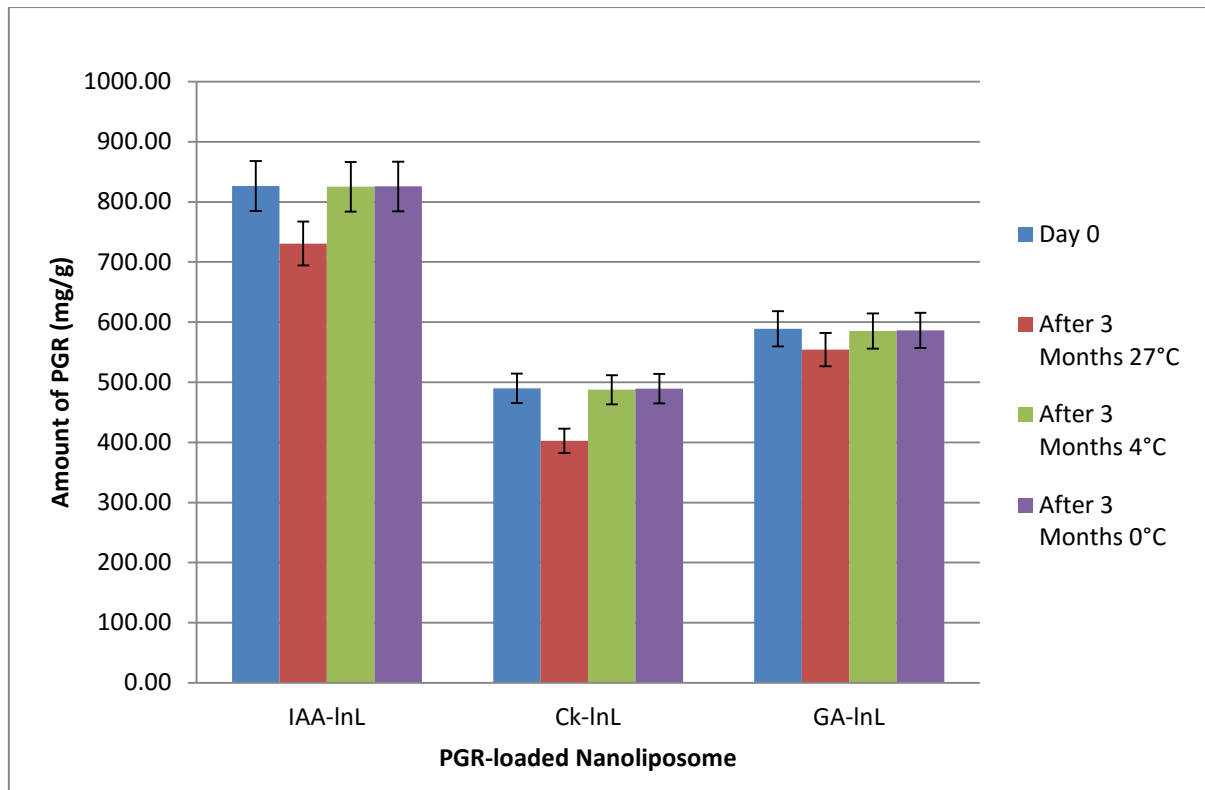**Figure 1:** Cumulative release profile of (A) IAA-loaded liposome, (B) Ck-loaded liposome and (C) GA-loaded liposome

Figure 2: Storage Stability Profile

Table 3: Amount of PGR content in liposome at day 0 and after 3 months stored at 27°C, 4°C and 0°C

PGR Samples	PGR Amount (mg/g)			
	Day 0	After 3 Months		
		27°C	4°C	0°C
IAA-IL	826.43a	730.79b	825.12a	825.62a
Ck-IL	489.66a	402.67b	487.53a	489.28a
GA-IL	588.90a	554.46b	585.14a	586.31a
Means in a row followed by a common letter are not significantly different.				

3.7 Effect of PGR-loaded Liposome on Makapuno Plumular Callus Initiation

In the study, Makapuno (*Cocos nucifera* L. var Makapuno) of VMAC 2 cultivar (NSIC 2008 Co-28) was used. VMAC 2 is one of the first developed makapuno hybrids.

Relative to color, shape and frequency, the callus formed in the medium supplemented with 100% IAA-loaded liposomes gave the highest acceptable quality scoring with initial callus formation after 8 days following culture (Figure 3). Formulation 1, 7, 14 and 15 also gave acceptable quality scoring based also on the three responses (Table 4). Formulation 6 and 14 also formed initial callus at an early stage of 8 days.

Table 4: Response in terms of color, shape, frequency and days to callus initiation of the makapuno plumule explants on varying ratio of PGR-loaded liposomes

FORMULATION	COMPOSITION (Independent)			RESPONSE (Dependent)			
	X1 – IAA	X2 – Ck	X3 – GA	Y1 –Color	Y2 – Shape	Y3 – Frequency (%)	Y4 - DTCI*
1	0.25	0.50	0.25	4.3	4.1	86.7	18.0
2	0.50	0.50	0.00	3.9	3.2	86.7	10.0
3	0.00	1.00	0.00	0.0	0.0	0.0	NA
4	0.00	0.75	0.25	3.9	3.1	42.9	15.0
5	0.00	0.50	0.50	4.8	3.0	53.9	22.0
6	0.75	0.25	0.00	4.3	4.0	64.3	8.0
7	0.25	0.25	0.50	4.6	3.7	92.3	22.0
8	1.00	0.00	0.00	4.9	4.9	100.0	8.0
9	0.50	0.25	0.25	0.0	4.0	78.6	15.0
10	0.50	0.00	0.50	4.5	3.9	60.0	15.0
11	0.00	0.25	0.75	4.5	3.0	80.0	25.0
12	0.25	0.00	0.75	4.7	3.7	71.4	20.0
13	0.00	0.00	1.00	4.6	0.0	0.0	NA
14	0.75	0.00	0.25	4.1	4.1	78.6	8.0
15	0.25	0.75	0.00	4.7	3.3	86.7	18.0

Callus initiation was observed after 8 days of plumule explants inoculation, and it continued to grow up to 10 days. Callus starts to grow from the explants and this was referred to as initial callus which has characteristic beige to white color and is hard [19]. Some treatments were able to form initial callus after 10 – 25 days. Of the 15 treatments, 8 gave an initial callus formation at higher frequency (> 75%). These treatments usually contain IAA-loaded liposomes and a mixture of IAA-loaded and cytokinin-loaded liposomes.

As shown in the contour and surface plots for color, shape and frequency (Figure 4), IAA-loaded liposome has a greater effect compared to the two other PGR-loaded liposomes. It further suggests that relative to shape and frequency, continuous increase in the amount of Ck-loaded and GA-loaded liposome gave no acceptable response and majority of the response were attributed to the IAA-loaded liposome in the medium.

Formation of translucent structure was also observed in some treatments (Figure 5). The presence of these translucent structures in some formulations suggests that the PGR-loaded liposomes, specifically the 100% IAA-loaded liposome and mixture of 75% IAA-loaded liposome and 25% cytokinin-loaded liposome, is capable of forming embryonic callus at earlier stage. Moreover, small globular callus was also observed in formulation 8 denoting a further formation of embryonic callus.
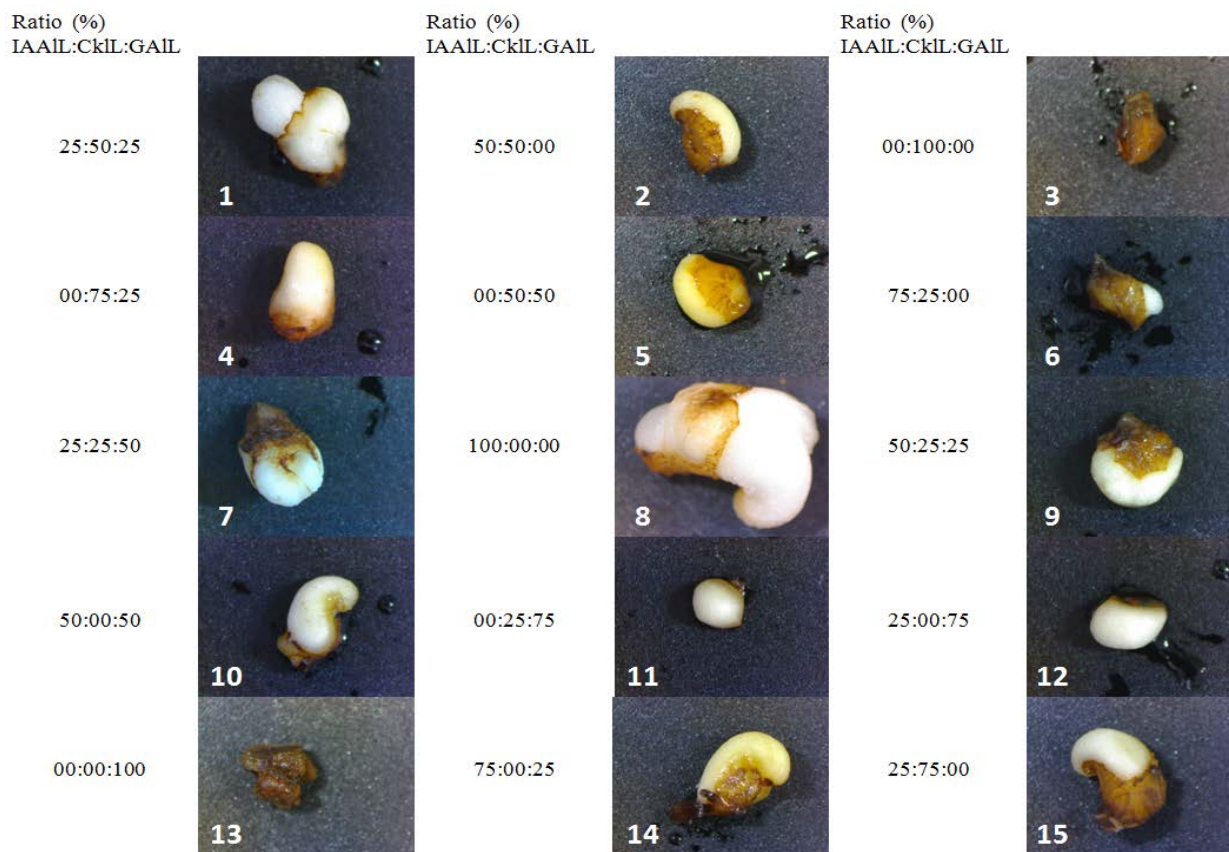
Figure 3: Representative samples from each treatments after 1 month incubation

Comparative testing was also done with the 100% IAA-loaded liposome formulation to an unencapsulated bacteria-derived crude auxin extract, phospholipid and water only (Table 5).

Table 5: Effect of free and encapsulated IAA on color, shape frequency and days to callus initiation of makapuno plumule explants

Treatment	Color	Shape***	Frequency*	DTCI***
Water	---	---	---	---
Phospholipid	---	---	---	---
IAA-lnL	4.9 ± 0.4	4.9 ± 0.4	100 ± 0	8 ± 1
Free Crude Auxin	4.7 ± 0.4	4.1 ± 0.3	98.2 ± 3.1	20 ± 2

--- Indicates no result or no reaction

DTCI - Days to callus initiation

Results are presented as mean (n=15) ± standard deviation

*** Highly significant
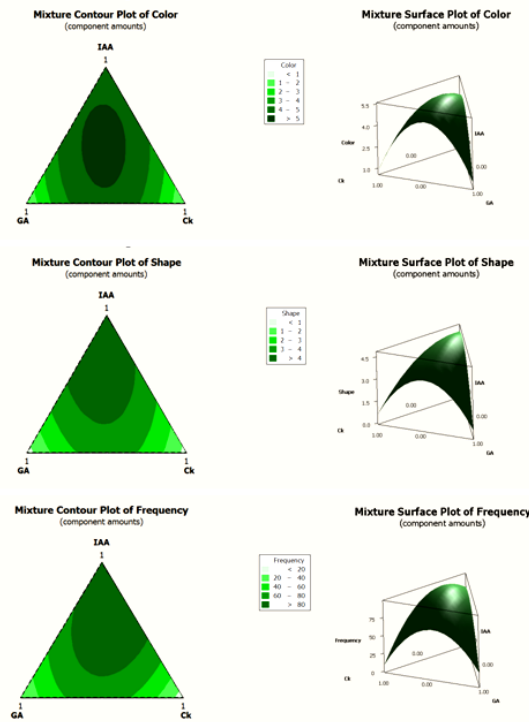
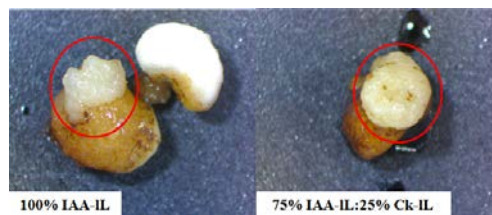**Figure 4:** Contour and surface plot of color, shape and frequency showing the maximum response in dark green color for the contour plot and elevation for surface plot

Figure 5: Initial callus forming translucent structure (encircle in red)

No significant differences were observed in the color ($P > 0.05$) of the initial callus formed from the encapsulated and unencapsulated crude auxin. However, significant differences were observed in the shape of the initial callus, frequency and days to callus initiation ($P < 0.05$). IAA-loaded liposomes gave the most initial callus formed at an early stage of 8 days while the unencapsulated crude auxin, though still high in frequency, initial callus formation was observed after 20 days following culture. This suggests that the encapsulated form of the IAA can facilitate faster absorption to the explants thus initiating callus at early stage with good shape and at higher frequency.

Using statistical software to analyze design of mixtures, the optimized composition of the composite was predicted to be 70.71% IAA-loaded liposomes, 8.10% Ck-loaded liposomes and 21.19% GA-loaded liposomes with desirability of 97.43%. However, though the predicted components have high desirability value, still the frequency of initial callus formation is low (88.16%) which is quite undesirable for large-scale micropropagation of coconut.
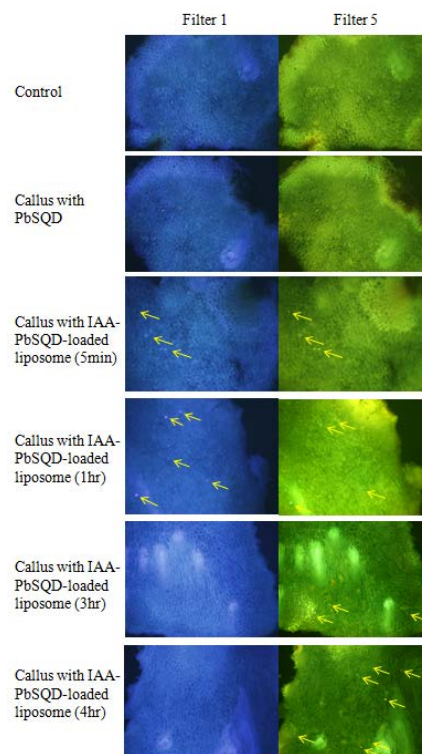
3.8 Uptake of IAA-loaded Liposome-PbSQD Hybrid on Makapuno Initial Callus

The fate of the IAA-loaded liposome was monitored using PbS quantum dots. Fabrication of the IAA-loaded liposome and quantum dots followed the lipid-film hydration method.

In the study, IAA-loaded liposome with QDs was supplemented to the Y3 medium where the Makapuno initial calli were incubated for 5 hours (Figure 7). Epifluorescent/light microscope photograph shows that entry of the tracer was facilitated even after 5 minutes of incubation, as observed by few fluorescent signals.

The fluorescence continues to be visible after one hour of incubation and has almost no distinct difference after the third and fifth hour of incubation. This may also be due to the very low concentration of the tracer used and/or may possibly be due to the thickness of the calli specimen under observation which may lead to the masking of the tracer.

A more highlighted specialized tissue was observed, with distinct fluorescence, after 3 and 5 hours of incubation. This suggests a localization of the tracer used. There was also fluorescence that can be observed at filter 5 but almost not visible under filter 1. The uptake of the IAA-loaded liposome with PbS QDs was not influenced by the QDs since calli treated with QD only does not have any fluorescence as shown in Figure 7. Untreated initial callus were also included for comparison of the autofluorescence of the sample without any tracer.

Figure 7: Epifluorescent/light microscope photograph of IAA-loaded liposome-PbSQD hybrid in 1 month-old Makapuno initial callus where fluorescence are pointed with an arrow (yellow)

4. Discussion

Bacteria synthesize auxins, and other plant growth regulators, in order to affect host physiological processes for their own benefit [20]. Plant growth regulators production is the major property of the bacteria colonizing the rhizosphere which stimulates, facilitates and enhances plant growth and that is why they are referred as plant growth-promoting rhizobacteria (PGPR) or plant growth-promoting bacteria (PGPB) [21]. Isolation of possible plant growth regulators from bacteria is very important since plant and rhizobacteria interaction can be unstable [22] where good results in vitro cannot be reproduced dependably under field condition [23]. This suggests high practical applications of the isolated PGR in field. The low active component of Ck and GA may be due to the extraction process or it may suggest that another form of Ck and GA could be present in the crude extract. The low activity of GA could also be possible due to the unpurified extracts characterized by its distinct liquid characteristics unlike with the crude IAA and cytokinin.

Liposomal membranes consist of amphipatic molecules which are oriented in a manner that their hydrophilic ends will be towards the aqueous environment. Phospholipids are the most abundant of these membrane molecules, which have a cylindrical shape, with the cross-sectional areas of the hydrophilic and hydrophobic portions being roughly equal [24]. The isolated phospholipids from soya lecithin contain more than 50% phosphatidylcholine (PC), about 25% phosphatidylethanolamine (PE) and less than 2% lysophosphatidylethanolamine (lyso-PE). Wu and Wang [10] reported 73, 24, and 3% of phosphatidylcholine, phosphatidylethanolamine and phosphatidyl inositol (PI) respectively. This suggests that the phospholipid used in the study is PC-enriched and also has a 29.89% recovery. Several nanoencapsulation have been employed in food and drug industry utilizing rice- and soy lecithin-derived phospholipids in its crude or purified form (phosphatidylcholine) [25].

When amphiphilic molecules such as phospholipids are placed in aqueous solutions, it will form vesicles in an attempt to shield its hydrophobic end while maintaining a close contact with water via its hydrophilic head [13]. This suggests that considering the insolubility of the crude PGRs in water, it has the greater chance of binding in the hydrophobic end of the phospholipid during the resuspension process. With enough energy introduced to the phospholipid aggregates, it will further organize to a close bilayer vesicle, which is why the resuspended MLVs were sonicated. Upon sonication, an ample amount of PGRs were secured in the inner portion of the vesicles.

Beta-sitosterol was also used in the fabrication of the liposomes as stabilizing agent for the vesicles. Mozafari and Mortazavi [26] reported that though phosphatidylcholine is capable of forming vesicles, still there is no assurance that it is very stable due to geometrical constraints. They further reported that this constraint will be addressed by adding stabilizing agents such as sterols. The most common sterol used in liposome industry is cholesterol, but phytosterols is also used especially in dealing with plant application. Since cholesterol is not generally or almost negligibly present in plants, β-sitosterol was utilized in the study. Generally, sterols modulate fluidity of phospholipid membranes by providing steric hindrance to the movement of the bilayer and preventing crystallization of the acyl chains of phospholipids which contributes to the stability of liposomes and reduces the permeability of the lipid membrane to solutes. In this case, chances of having leakage of the PGRs will be lowered. The amount of sterol added is dependent on the application of the liposome. Liposome

containing 10% cholesterol was able to incorporate DNA in the presence of calcium ions. However, increasing the level up to 40% showed no interaction with the membrane and was not able to incorporate DNA, concluding that 40% and above cholesterol composition is not suitable for drug delivery application [27]. In the study, the amount of β-sitosterol added is 1/3 of the weight of the phospholipid used for further stability and to insure incorporation of the plant growth regulators.

Encapsulation efficiency is a very important index in active compound delivery system. The percent encapsulation efficiency of the samples was somehow correlated to its lipid-to-PGR ratio. The more the PGR present, in a ratio, upon vesicle formation, the more it is likely to be loaded in the liposome such as in the case of the IAA-loaded (3:1) and Ck-loaded (2:1) liposomes which was also observed by Colletier and his colleagues [28]. Panwar and his colleagues [15] reported an encapsulation efficiency range of 50 – 72% for the nanoencapsulation of albendazole. An encapsulation efficiency range of 86 – 93% was also reported for the liposomal encapsulation of quercetin and resveratrol [29].

The bigger size observed for the samples may be due to the storage condition of the samples. In the study, samples were stored in a dessicator at room temperature (approx. 25°C) for almost a month prior to analysis. Based on the storage stability test on this study, samples stored at about 27°C tend to have a decrease in active component that may be attributed to the increase in permeability of the liposomes and form aggregates.

Standard plant growth regulators are known to be insoluble in water in which NAOH and EtOH are sometimes used to partially dissolve these compounds in preparation of medium for tissue culture. Result showed an improved solubility of the bacteria-derived PGRs in liposomal form under aqueous solution. Liposomal-assisted solubility was also reported in previous researches on nanoencapsulation [28,30].

Higher PGR amount were released at pH 5.0 than at pH 7.0 which implies an increase in the permeability of the liposome. The increased PGR released at acidic pH could be possibly because of the increased fusion of liposomes due to acidification which was also reported by Osanai and Nakamura [31]. Low pH may disrupt the membrane bilayer and hence make the membrane leaky releasing PGR into the surrounding medium similar to the phenomenon observed by Drummond and his colleagues [32]. On the other hand, Ho and his colleagues [33] reported less than 1% hydrolysis of PC at an extreme pH of 1 in about 4 h whereas it was stable to hydrolysis in the pH range of 4–10. The literature reports that in vitro release profiles from liposomes characteristically show an initial fast drug loss followed by slower rates of drug loss [34][35]. The observed initial fast rate of release may be due to the detachment of the active component from the liposomal surface while the later slow release results from sustained active component release from the inner lamellae. The result further suggests that it takes time for the PGRs to be released once encapsulated in the liposomes because lipid bilayer was stabilized by the β-sitosterol as observed also by Panwar and his colleagues [15]. It has been reported that at elevated temperatures, phospholipids undergo a phase transition that alters their permeability [13] leading to a temperature-assisted leakage of the active component. This could also be due to the oxidation of the unsaturation located in the lipid chain of the phospholipids as reported by Batista and his colleagues [36].

In vitro regeneration of coconut (*Cocos nucifera* L.) in general, is difficult to do. This serves as a threat since

nowadays demand for coconut is high either by industrial or for breeding purposes to be able to create a better cultivar. In the Philippines, coconut planting material is highly in demand since most of the existing coconuts were devastated by the scale insect leading to serious effect on the economics of coconut farmers. Several coconut tissue culture protocols had been reported for the past few years utilizing different explants which include the plumule.

For plumule explants, and even somatic embryos, initial callus developed from the peripheral tissues as has been reported for other species such as *Vitis rupestris* S. [37] and *Quercus suber* L. [38]. Plumule explants cultured in medium containing 2,4-D, without changing the medium for 3 months, formed callus along the outer leaf primordial surrounding the shoot meristem within 15–30 days following culture [16].

Callus initiation was also reported by Bhagya and Chandrashekar [39] from *Cyclea peltata* (Lam) Hook. F. Thoms. planted in medium containing IAA or NAA alone and in combination BAP or Kinetin, further reported that the number of calli formed was significantly high in both single-hormone or mixed-hormone content. It is suggested that the IAA-loaded liposomes plays a significant role in the formation of initial callus from plumule explants, moreover, the encapsulation nature of the particulate makes it more easily available within the plant material thus forming the initial callus at earlier days compared to previous reports. Perez-Nuñez and his colleagues [16] also reported that as initial callus eventually evolved into embryogenic callus, several developmental changes were observed including the formation of ear-shaped translucent structures that appeared after 45 days. This will further form embryogenic callus and then somatic embryos with proper changing of medium.

The fluorescence of QDs is due to the excitation and relaxation of the electrons in the central core atom. When an electron is excited into the conduction band, it is unstable due to the large amount of energy and immediately returns to the lower energy valence band or ground state. As the electron back down, it releases photon in the form of light with an energy that matches the energy of the band gap. This emission is known as fluorescence. Fluorescence of the fabricated tracer in buffer solution (pH 7.2) was assessed using epifluorescent/light microscope and uptake of the IAA-loaded liposome was successfully evaluated using this technique.

5. Conclusion

Therefore, encapsulation of bacteria-derived plant growth regulators auxin, cytokinin and gibberellin enhances its solubility in water and stability. It further showed a controlled-release mechanism. Moreover it showed better bioactivity in the formation of initial callus in Makapuno (*Cocos nucifera* L. var Makapuno) plumule s. Lastly, the IAA-loaded liposomes were able to enter the tissue faster than the unencapsulated IAA.

6. Recommendations

For further improve the study it is highly recommended to use other extraction techniques for the isolation of the plant growth regulators in the bacteria and perform other bioactivity testing of the PGR-loaded liposomes, either in composite or individual, such as effect on rooting for the cuttings and/or effect on seed germination of high valued crop.

Acknowledgements

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