Development of Arbuscular Mycorrhizal Fungi-Organic Fertilizer Pellets Encapsulated with Alginate Film

Article *in* Engineering Journal · December 2018

DOI: 10.4186/ej.2018.22.6.65

CITATIONS
11

READS
848

5 authors, including:

Jeerun Kingkaew

6 PUBLICATIONS **109** CITATIONS

SEE PROFILE

Suriyan Cha-Um

National Science and Technology Development Agency

196 PUBLICATIONS **4,076** CITATIONS

SEE PROFILE

Muenduen Phisalaphong

Chulalongkorn University

96 PUBLICATIONS **2,975** CITATIONS

SEE PROFILE

Engineering Journal issn: 0125-8281

Article

Development of Arbuscular Mycorrhizal Fungi-Organic Fertilizer Pellets Encapsulated with Alginate Film

Pornpicha Pitaktamrong[1,a], Jeerun Kingkaew[2], Suravoot Yooyongwech[3], Suriyan Cha-um[4], and Muenduen Phisalaphong[1,b,*]

1 Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok, Thailand
2 Food Technology Program, Mahidol University, Kanchanaburi, Thailand
3 Department of Agricultural Science, Mahidol University, Nakon Pathom, Thailand
4 National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathum Thani, Thailand
E-mail: [a]pornpichapalm@gmail.com, [b]muenduen.p@chula.ac.th (Corresponding author)

Abstract. A novel formulation consisting of arbuscular mycorrhizal fungi (AMF) spores mixed with sterilized organic fertilizer (AMF-F) encapsulated by an insoluble calcium alginate film was developed to enhance AMF efficacy and stability. The hardness of the pellets increased from 7–8 N to approximately 80 N by increasing the alginate concentration of the coating film from 1 to 3%. The AMF spore germination rate for the AMF and AMF-F pellets coated with calcium alginate films depended on the alginate concentration. A 2% sodium alginate formulation for the coating films resulted in optimal AMF spore germination rates and mechanical properties for handling, transport, and stability. The inclusion of a sterilized organic fertilizer in the encapsulated AMF-F pellets considerably induced AMF mycelial growth and helped prolong the shelf life of the pellets. In soil, the AMF-F pellets encapsulated with alginate (cAMF-F) initially degraded faster than the alginate-encapsulated AMF pellets (cAMF). However, both types of pellets were fully degraded within 30 days. It was demonstrated that cAMF and cAMF-F could promote colonization and provided resistance to drought stress in maize potted plants.

Keywords: Arbuscular mycorrhizal, organic fertilizer, alginate, encapsulation, pellets.

ENGINEERING JOURNAL Volume 22 Issue 6
Received 15 March 2018
Accepted 20 September 2018
Published 4 December 2018
Online at http://www.engj.org/
DOI:10.4186/ej.2018.22.6.65

DOI:10.4186/ej.2018.22.6.65

1. Introduction

Climate change can lead to below average rainfall or aberrations in seasonal rainfall patterns, thereby decreasing the accumulated rainwater over time [1]. The effects of droughts on the environment are a serious concern, particularly for those involved in the commercial cultivation of agriculturally important crops. Thailand experiences drought conditions almost annually, and the resulting adverse consequences for the agricultural sector include decreased crop productivity, especially in rice fields [1]. Drought stress inhibits the ability of plants to absorb nutrients (e.g., nitrogen and phosphorus) [2]. Organic fertilizers are eco-friendly products that may be useful for maintaining the suitability of agricultural lands for crop production, especially in organic farming systems. Furthermore, many types of soil microbes can fix phosphorus, which may enhance the ability of plants to take up this important nutrient. Arbuscular mycorrhizal fungi (AMF) are plant growth-promoting organisms [3]. These fungi adsorb mineral nutrients from soil and transfer them to plants. They can penetrate vascular plant root cortical cells and help increase the surface area of the root system [3]. The AMF can act as nutrient transporters between the soil and plants, and may be particularly useful for transferring phosphorus [3, 4]. Symbiotic associations of AMF on plant roots have several beneficial effects on the host plant, soil, and environment. These associations improve plant nutrient uptake, especially for phosphorus, which can be transferred much faster in mycelia than in roots [3]. Additionally, AMF help increase soil structure stability and decrease the effects of drought conditions on host plants because their mycelia can increase the absorption range of roots [5-7]. Mycorrhizal colonizations and arbuscles also have a major role in enhancing the metabolism of reactive oxygen species in drought-stressed plants to minimize oxidative damage [8]. In addition, AMF has been reported for protection against Al toxicity [9] and assisting heavy metal hyperaccumulators in the remediation of contaminated soils [10]. Products containing AMF are increasingly being used worldwide [11]. From our preliminary test of AMF culture, the colonization (%) of AMF was evidently observed in the root of maize and rice plants inoculated with the AMF culture. In addition, we also found significant improvement in plant height (cm), leaf number and leaf area of maize and rice plants inoculated with the organic fertilizer and AMF culture. However, water loss and soil toxicity might inhibit the germination of AMF spores. Therefore, an encapsulation technique has been developed to protect AMF from adverse environmental conditions. Additionally, encapsulation can be used to delay the release of nutrients from fertilizers and increase the hardness of fertilizer granules [12-15]. Many types of materials have been used for the encapsulation of granular fertilizers, including polysulfone, polyacrylonitrile, cellulose acetate [12, 15], starch/PVA [13], and natural biopolymers (e.g., agar and sodium alginate) [14]. The properties of the encapsulated granules mainly depend on the type and concentration of coating materials. Alginate is a natural polymer extracted from brown seaweed. It is generally regarded as safe and is commonly used because it is nontoxic to humans and the environment, inexpensive, and suitable for all types of microbes. Alginate has a high water absorption capacity (WAC). Furthermore, it can be gelled into hydrophilic matrices and crosslinked under mild conditions, enabling cell entrapment with minimal loss of viability [16]. Calcium chloride ($CaCl_2$) is often used as a crosslinking agent for sodium alginate. Alginate encapsulation may be relevant for the delivery of fungi to toxic waste sites [17].

In this study, we developed and evaluated a novel approach to encapsulating AMF-organic fertilizer (cAMF-F) pellets with alginate film to improve AMF efficacy and stability, especially under drought conditions. A film-coating technique was developed to protect AMF spores from toxic environments and prolong their shelf life. .

2. Materials and Methods

2.1. Materials

A stock AMF culture in soil consisting of *Glomus* sp. and *Acaulospora* sp. spores (1:1) in the concentration of 149.3 ± 2.9 spores per 25 g soil was kindly provided by the Department of Agriculture, Ministry of Agriculture and Cooperatives (Bangkok, Thailand). Organic fertilizer was purchased from Charoen Pokphand Northeastern Public Co., Ltd. (Bangkok, Thailand).

2.2. Preparation of Sodium Alginate and Calcium Chloride Solutions

Powdered sodium alginate (Acros Organics, USA) was dissolved in deionized (DI) water at 60°C to prepare 1, 2, and 3% (w/v) solutions. The solutions were thoroughly mixed until they became clear. A 0.1 M $CaCl_2$ solution (Ajax Finechem Pty. Ltd., Australia) was prepared in DI water and then used as a crosslinking agent.

2.3. Preparation of cAMF and cAMF-F Pellets

The stock AMF culture in soil was thoroughly mixed with glycerol (10 g stock AMF culture: 1 ml glycerol). After that, the mixture was compressed into cylindrical pellets of 0.7 cm diameter and 0.5 cm thick by a manual hand press machine (Bangkok, Thailand). The compressed AMF pellets were then immersed in the prepared 1, 2, or 3% (w/v) alginate solutions, after which they were cross-linked by soaking in the 0.1 M $CaCl_2$ solution for 30 min and then air-dried at room temperature for 24 h. A subsequent analysis of the resulting AMF pellets coated with alginate film (cAMF pellets) revealed they consisted of approximately 15% water. To prepare AMF-F pellets coated with alginate (cAMF-F pellets), the stock AMF culture in soil was mixed with sterilized organic fertilizer (1:1 by weight) and then combined with glycerol (10 g AMF-F: 1 ml glycerol). The AMF-F pellets were fabricated, coated, cross-linked, and dried as described for the preparation of the cAMF pellets. The cAMF and cAMF-F pellets were stored in plastic bags in a dark cabinet at room temperature (29±2 °C).

2.4. Characterization of cAMF and cAMF-F Pellets

Morphologies of surface and cross section of the cAMF and cAMF-F pellets were measured by Scanning Electron Microscope (SEM). The pellets was rapidly cut by a sharp blade, sputtered with gold and photographed. The coated specimens were examined under SEM using a JOEL JSM-5410LV microscope (Tokyo, Japan).

The mechanical property of the pellets was determined in term of hardness. The hardness of the cAMF and cAMF-F pellet was measurement by using universal testing machine (model ES-Z, Shimadzu, Japan). The hardness was reported as the average value determined from seven pellets (n = 7).

Water absorption capacity (WAC) of AMF and AMF-F pellets was expressed as grams of adsorbed water per grams of dry pellet:

$$\mathrm{WAC} = \frac{W_w - W_d}{W_d} \times 100\%$$

where, W_w and W_d are the weight of a wet pellet and dry pellet, respectively. WAC was reported as the average value determined from fourteen pellets (n = 14).

2.5. Germination of Fungal Spores on cAMF and cAMF-F Pellets

The germination of fungal spores on the cAMF and cAMF-F pellets was observed on glass plates containing Whatman® Cellulose Membrane No. 1 (11 μm diameter pores) (Sigma-Aldrich, USA) moistened with DI water. The pellets were incubated at 30–32°C for 11 days, and then analyzed using an SMZ-161 stereomicroscope (Motic, Japan). The germination rate (%) was calculated based on the average value for fifty pellets (n=50). Germination in this experiment means any mycelia seen on pellets had been counted as 1.

2.6. Nutrient Release Rate for cAMF and cAMF-F Pellets

The nutrient (N, P, and K) release rate at 30°C was calculated as a function of time. Specifically, 1 g cAMF or cAMF-F pellets was placed in a plastic tube containing 50 ml DI water. An aliquot of the solution was collected without agitation every 6 h up to 48 h. The nutrient release rate was estimated based on the accumulated released nutrients (i.e., NH_4^+, K^+, Mg^{2+}, Ca^{2+}, NO_3^-, and PO_4^{3-}) analyzed by HPLC as described by Tanaka et al. (1999) [18] and Hossain et al. (2006) [19]. The reported value of each data point was from the average value of three samples (n=3).

2.7. Decomposition of cAMF and cAMF-F Pellets

The decomposition of the biodegradable cAMF and cMF-F pellets in soil was evaluated at room temperature (29±2 °C) for one month. The samples of cAMF and cAMF-F pellets (10 beads for each 8″ pot) were added into soil at about 3 cm below soil surface in each pot under uncontrolled composting conditions. Each sample was weighed every three days for one month or until it was completely degraded. For the calculation of weight loss, the sample was carefully taken out and soil on the sample surface was removed. The sample was then dried at 50°C for 24 h and then weighed. The biodegradation based on the mass loss was calculated according to the following equation:

$$\text{Biodegradation (\%)} = \frac{W_1 - W_2}{W_1} \times 100$$

where W_1 is the initial dry weight of the samples and W_2 is the dry residual weight of the samples after the decomposition in soil.

2.8. Stability of Stored cAMF and cAMF-F Pellets

The cAMF and cAMF-F pellets were kept in plastic bags maintained in darkness at 30–35 °C for 180 days to investigate the effects of storage. After that, the germination of fungal spores on the cAMF and cAMF-F pellets was evaluated at the same method previously described in Topic 2.5.

2.9. Effects of cAMF and cAMF-F pellets on plants

The maize (*Zea may L.*) seedling was grown in sterilized soil to test with the cAMF and cAMF-F pellets. After one week of plant seeding, cAMF, cAMF-F pellets (25 beads for each 12″ pot) were added into soil (1-2 cm below soil surface) and then were mixed with surface soil in the pots. This method was modified from the usual application in order to make it easier and more efficient for the fertilizer application. In addition, this method can be applied after plant germination. For sterilized organic fertilizer (F) plant, the fertilizer of 50% by weight of total weight of pellet was added into soil and then was mixed with surface soil in the pot. At one month-old seedlings, percentage of AMF colonization in maize roots by trypan blue method was observed and calculated [20].

A preliminary test for positive effect of cAMF and cAMF-F pellets on drought stress tolerance was performed in maize potted plants under drought stress and evaluated in term of the change of water content of maize shoots. Soil water content (SWC) was calculated by the following equation [21]:

$$\text{SWC (\%)} = [(\mathrm{FW} - \mathrm{DW})/\mathrm{DW}] \times 100$$

where the fresh weight (FW) of the 5-10 cm deep soil was obtained from each water condition (well-watered control and water deficit stress). The dry weight (DW) was obtained after the soil had been kept in hot air oven at 85 °C for 4 days. The changes of water content of maize shoots were detected and calculated by the following equation:

$$\text{Reductions of water content (\%)} = \left[\left(\frac{\mathrm{WC}}{\mathrm{FW}}\right)_{\text{irrigation}} - \left(\frac{\mathrm{WC}}{\mathrm{FW}}\right)_{\text{water deficit}}\right] \times 100$$

where WC and FW are the water content of samples and the fresh weight of samples, respectively.

3. Results and Discussions

3.1. Scanning Electron Microscopy

Cell entrapment within alginate is one of the most widely studied because cell viability and activity are kept very high. Generally, the entrapment is made by mixing cells (or spores) with alginate solution and then cell entrapped in alginate bead is formulated by using cross linking solution, for an example, a formulation of a mixture of AMF spore and alginate solution by dripping the mixture into with $CaCl_2$ solution as a cross-

linking agent [22]. However, this technique has received less attention for practical use due to several drawbacks, such as low physical strength and severe mass transfer limitation [23, 24]. The problem of molecular diffusion through alginate gel was the dominant mechanism for oxygen transfer in the pellets. For aerobic organisms, particularly for fungi, oxygen plays a vital role in many aspects of cellular metabolism. Changes in dissolved oxygen often impact respiration rate, growth rate and activities. To avoid a problem of mass transfer limitation, the diameter of alginate bead was suggested to be not greater than 2 mm [25]. Consequently, large-scale production of these carriers often requires quite complex equipment leading to high cost of production.

In this study, a different technique for the fabrication of fertilizer pellets was developed; cAMF or cAMF-F pellets were made by direct compression of mixtures of the stock AMF culture in soil, organic fertilizer and glycerol before encapsulation with calcium alginate film. The coated fertilizer pellets could be separated into of two parts as shown in Fig. 1. The first part is a core part composed of the compressed mixture of AMF culture in soil, organic fertilizer and glycerol. The second part is a cover part made by coating AMF or AMF-F pellet with a thin film of calcium alginate (film thickness of about 0.1 and 0.2 mm for one layer-coating (1L) and two layer-coating (2L), respectively). The diameter and thickness of the cylindrical pellets were approximately 0.7 cm and 0.5 cm, respectively. There were no considerable differences in the overall appearance of the cAMF and cAMF-F pellets regardless of the alginate concentration of the coating solution. The surface and cross-section morphologies of the cAMF and cAMF-F pellets with one layer coating of 2% (w/v) alginate are presented in Fig. 1. Images of the pellet cross-sections revealed thin films of Ca^{2+}-crosslinked alginate that were approximately 0.1 mm thick. Because of the presence of the organic fertilizer, the surface of the cAMF-F pellets was slightly rougher than that of the cAMF pellets. The encapsulation by appropriate matrix could protect cells by fortification from toxins and inhibitors [23, 24]. By inoculating active AMF culture to plants, high germination and growth of AMF should be obtained, which it consequentially promotes good symbiotic association of AMF mycelium in the plant roots. As a result, the inoculation of AMF in form of c-AMF and c-AMF could help promote good symbiotic association of AMF mycelium in the plant roots and therefore, provide resistance to drought stress in plants.

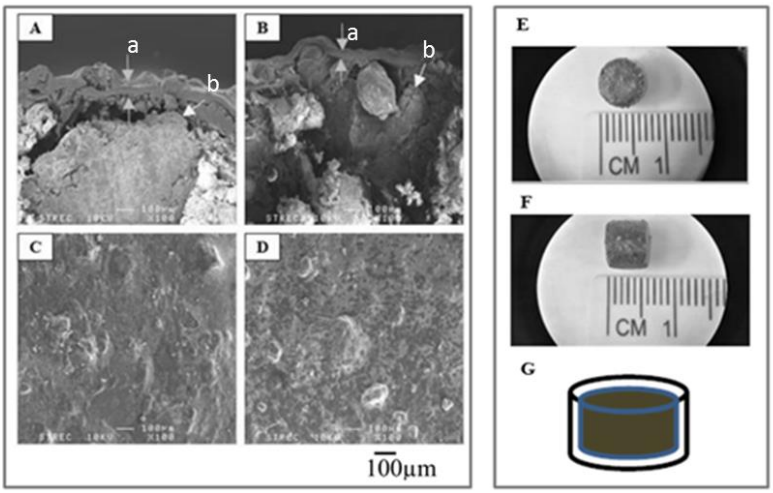

Fig.1. SEM images of pellets with a single coating film (1L) of 2 % (w/v) alginate: cross-section of cAMF (A) and cAMF-F (B); surface of cAMF (C) and cAMF-F (D); outlook of cAMF pellet (E, F) and illustration of pellet structure (G). The arrows at "a" and "b" positions show the coating film and inner pellets, respectively.

3.2. Hardness of cAMF and cAMF-F Pellets

Hardness refers to the resistance of solid matter to deformation or collapse when a compressive force is applied [26]. It is an important property that directly influences the transport, handling, and storage of materials. Details regarding the hardness of the cAMF and cAMF-F pellets are presented in Fig. 2A and 2B, respectively. Increasing the alginate concentration of the coating solution from 1 to 3% resulted in a 4-10 fold increase in pellet hardness. The hardness values of the cAMF and cAMF-F pellets coated with one layer

(1L) of 3% alginate film were 86.4 and 97.0 N, respectively. Adding an additional layer (2L) of alginate film increased the hardness of the cAMF and cAMF-F pellets to 145.4 and 179.8 N, respectively. To ensure the mechanical strength of the cAMF and cAMF-F pellets was appropriate for handling and transport, the alginate concentration in the coating solution needed to be at least 2%. It was demonstrated that the presence of the solid core coated with thin film of alginate gel outside could support and held the components together with great strength. Also, the entrapment by coating pellets of cells in soil/fertilizers with thin alginate film outside should avoid severe mass transfer limitation. In addition, this technique requires simple equipment and it is practical for large-scale production.

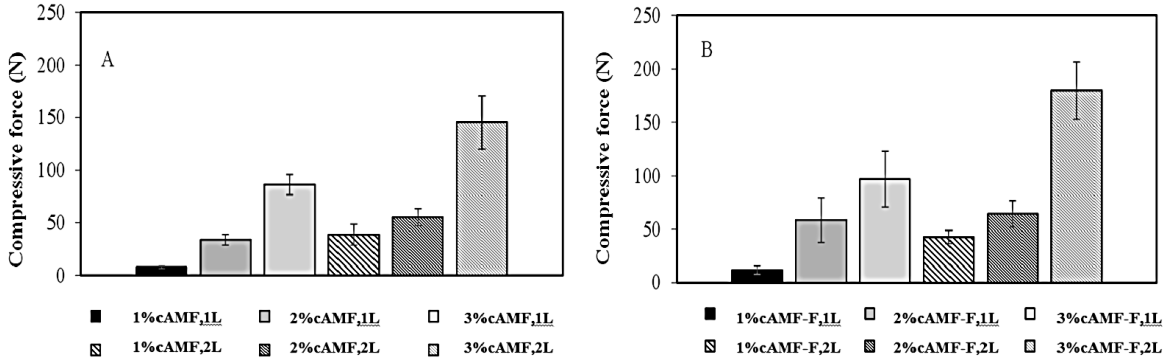

Fig. 2. Hardness of cAMF (A) and cAMF-F (B) pellets coated with 1, 2 and 3% (w/v) alginate films in forms of 1-layer (1L) and 2-layer (2L) films (mean±SE, n=7).

3.3. Water Absorption Capacity of cAMF and cAMF-F Pellets

The WAC is an important factor affecting fungal spore germination and mycelial growth as well as the nutrient release rate of the cAMF and cAMF-F pellets. The WAC of the pellets coated with one layer alginate film was relatively increased from 25-26% to 62-65% with the increase of alginate concentration in the coating film from 1% to 3% w/v and there were no significant differences between the WAC of cAMF and cAMF-F pellets (Fig. 3). However, pellets coated with two layers of alginate film had 13-60 % higher WAC than those of the pellets with only one layer, with larger differences at higher alginate concentrations. Increasing alginate concentrations in the coating solution resulted in greater swelling of pellets immersed in water. Alginate is very hydrophilic, and water molecules are easily absorbed into the alginate film. Previous studies confirmed that sodium alginate film is relatively unstable in aqueous solutions, and the degree of swelling in water and the WAC increase with increasing alginate concentrations [27, 28]. In this study, encapsulating AMF and AMF-F pellets with a relatively high alginate concentration or with two layers of alginate film enhanced the adsorption and retention of water.

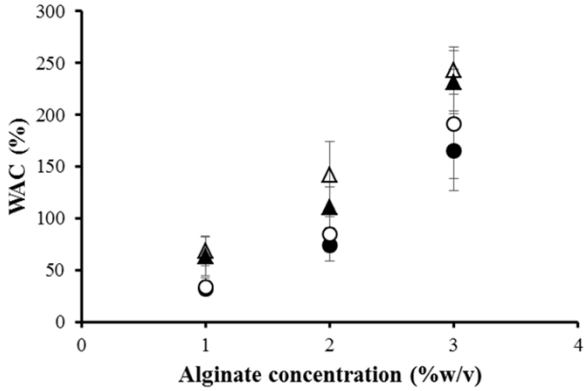

Fig. 3. WAC of cAMF and cAMF-F pellets coating with 1-3% (w/v) alginate: cAMF,1L (●); cAMF,2L (▲); cAMF-F,1L (○); cAMF-F,2L (Δ) (mean±SE, n=14).

3.4. Germination of Fungal Spores on cAMF and cAMF-F Pellets

Germination of fungal spores of the cAMF and cAMF-F pellets was tested at room temperature (30 – 32°C), which was average soil temperatures at the surface in Thailand. The spore contents of cAMF and cAMF-F pellets were about 60 and 30 spores per 10 g, respectively. Spore germination generally requires the presence of water and oxygen. It was shown that the germination rates of AMF spores of pellets coated with one layer of 1% alginate film were slightly lower (2% max), and the presence of organic fertilizer had no significant effect (Fig. 4). However, among pellets coated with 2 or 3% alginate, the initial AMF spore germination rate of cAMF pellets was higher than that of cAMF-F pellets, which was likely because cAMF pellets contained more spores. Additionally, the initial AMF spore germination rate was higher for pellets coated with two layers of alginate film than for pellets with only one layer. The higher AMF spore germination rate observed with 2% alginate pellet coatings than with 1% alginate coatings may have been due to the increased hydrophilicity of the coating film. However, the greater density of the coating at a relatively high alginate concentration (3%) may inhibit the transfer of oxygen across the film. The oxygen transfer across alginate gel has been found to decrease with increases in gel thickness and alginate concentration [29]. This may explain the lower AMF spore germination rate for the cAMF pellets coated with 3% alginate than for the cAMF pellets coated with 2% alginate. The growth of AMF mycelium on pellets coated with 2% alginate is presented in Fig. 5. The addition of organic fertilizer may not directly affect spore germination, but could help to promote mycelial growth on cAMF-F pellets. After the germination, rapidly growth of AMF mycelium of cAMF-F pellets was observed. At high relative humidity, only 3-4 days were required for the AMF mycelia to cover about half the outer surface area of granules. The entire outer surface area of the cAMF-F pellets was covered with mycelia after 5 days. On the other hand, relatively low mycelial densities were observed on cAMF pellets after the incubation for 5 and 7 days. The stimulation of mycorrhizal colonization and the promotion of hyphal growth by chemical factors have been previously reported [30]. It was found that a growth stimulant isolated from the brown alga increased hyphal growth of AM spores germinated in vitro [31]. In this study, it was suggested that hyphal or mycelial growth of AMF were improved in the presence of organic fertilizer as demonstrated on cAMF-F pellets.

3.5. Nutrient Release Rate

The nutrient release rate was influenced by the thickness and porosity of the coating materials. The total amounts of nutrients (PO_4^{3-}, K^+, NO_3^-, NH_4^+, Mg^{2+}, and Ca^{2+}) obtained from the cAMF and cAMF-F pellets are shown in Table 1, while Fig. 6 presents the accumulation of the primary (PO_4^{3-}, K^+, and NO_3^-) and secondary (Mg^{2+} and Ca^{2+}) nutrients released over time. Because of a lack of organic fertilizer, very few K^+ and almost no PO_4^{3-} were collected from cAMF pellets. In contrast, the PO_4^{3-} and K^+ concentrations obtained from cAMF-F pellets were about 2.5 and 2.3 mg/g, respectively. We observed that PO_4^{3-} was released slowly from cAMF-F pellets (i.e., between 6–48 h), whereas K^+ was released faster (i.e., within 12 h). The concentrations of NO_3^- and NH_4^+ obtained from all pellet types were ≈ 0.1–0.3 mg/g. Similar to K^+, NO_3^- and NH_4^+ were released from pellets within 12 h. The accumulated NH_4^+ content decreased significantly after 12 h, likely because of decomposition.

Because $CaCl_2$ was used as the crosslinking agent, the cAMF and cAMF-F pellets contained relatively high Ca^{2+} concentrations (i.e., approximately 2–3 mg/g). Mg^{2+} and Ca^{2+} in cAMF pellets was released rapidly and almost completely within 12 h, whereas Mg^{2+} and Ca^{2+} in cAMF-F pellets were totally released within 48 h. This should be because the total concentrations of these secondary nutrients were considerably higher in cAMF-F pellets. Since the organic fertilizer used in this study contained many plant nutrients at relatively higher levels than those in the stock soil culture, cAMF-F pellets contained higher levels of certain plant nutrients.

The number of layers of alginate film did not significantly affect the nutrient release rate. Whether one or two layers were applied, the pellet coating decreased the release rate of the primary and secondary nutrients. A lack of coating reportedly results in considerable losses of fertilizer to the environment, which may eventually contaminate water supplies [13]. Fertilizer nutrient release rates are generally dependent on the materials and methods used to encapsulate the fertilizer granules, as well as the nutrient contents and soil conditions. Previously, capsules made of alginate gelatinized with Ca^{2+} have been used to control the release of drugs, pesticides and chemical and bio-fertilizers [32-35].

DOI:10.4186/ej.2018.22.6.65

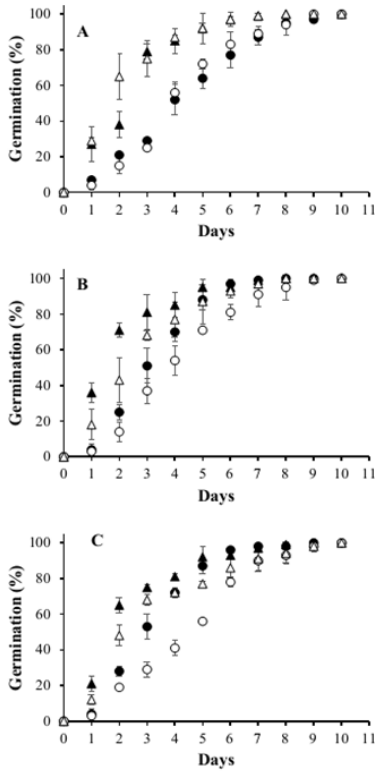

Fig. 4. Germination percentages of AMF mycelium from cAMF and cAMF-F pellets coated with different concentrations of sodium alginate at 1% (A), 2% (B) and 3% (C): cAMF,1L (●); cAMF, 2L (▲); cAMF-F,1L (○); cAMF-F,2L (Δ) (mean±SE, n=50).

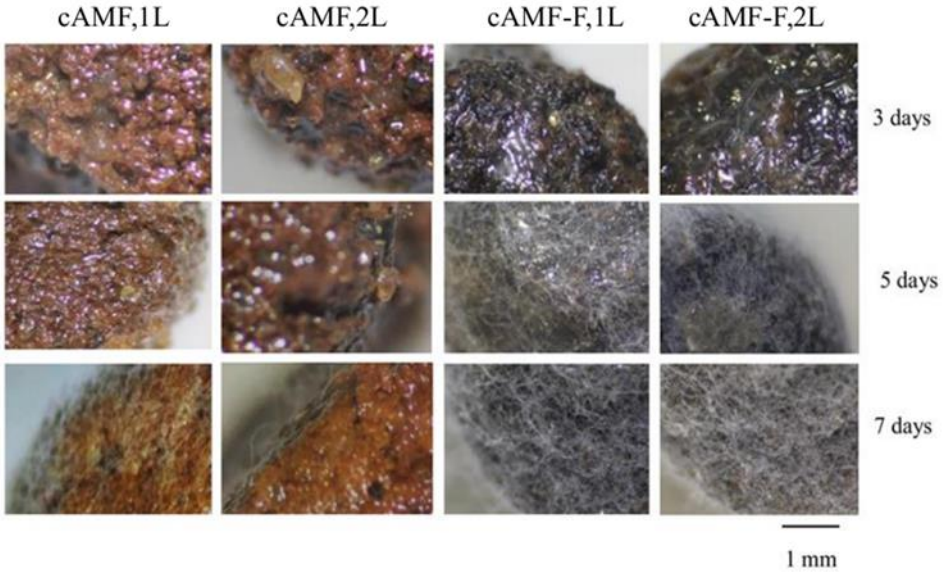

Fig. 5. Growth of AMF mycelium on cAMF and cAMF-F pellets coated with 2% alginate films (1L and 2L) after the incubation for 3, 5 and 7 days.

Table 1. Total amount of nutrients (PO_4^{3-}, K^+, NO_3^-, NH_4^+ , Mg^{2+} and Ca^{2+}) obtained from cAMF and cAMF-F pellets (mean ±SE, n = 3).

Total amount of nutrients	PO_4^{3-} (mg/g)	K^+ (mg/g)	NO_3^- (mg/g)	NH_4^+ (mg/g)	Mg^{2+} (mg/g)	Ca^{2+} (mg/g)
cAMF	0.00±0.00	0.44±0.02	0.21±0.04	0.11±0.04	0.09±0.01	1.88±0.16
cAMF-F	2.54±0.12	2.33±0.74	0.23±0.03	0.27±0.10	0.82±0.12	3.14±0.10

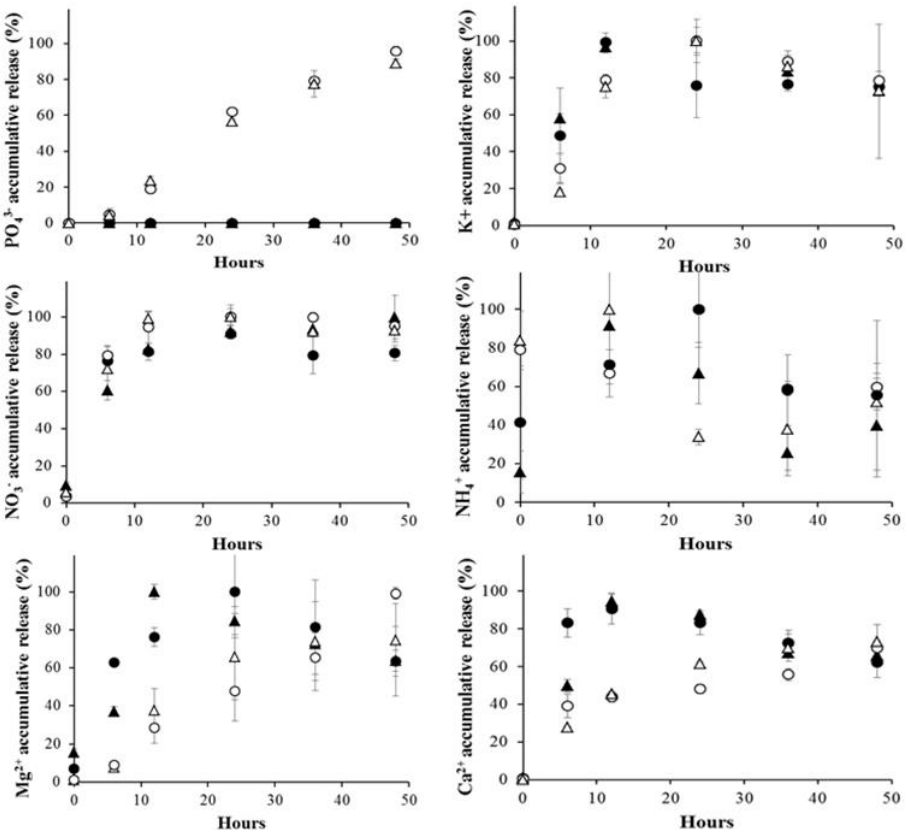

Fig. 6. Accumulative releases of nutrients (PO_4^{3-}, K^+, NO_3^-, NH_4^+ , Mg^{2+} and Ca^{2+}) from pellets coated with 2% alginate films: cAMF,1L (●); cAMF,2L (▲); cAMF-F,1L (○); cAMF-F,2L(Δ) (mean±SE, n=3).

3.6. Decomposition of cAMF and cAMF-F Pellets

The decomposition of the biodegradable cAMF and cMF-F pellets in soil was evaluated (Figure not shown). The cAMF-F pellets were highly biodegradable, with ~50% of the pellets degraded within 9 days in soil. The number of alginate film layers had no significant effect on the decomposition rate. The cAMF pellets degraded more slowly than the cAMF-F pellets. Thus, the organic fertilizer appears to affect the decomposition rate. As indicated in Table 1, the nutrient content (e.g., PO_4^{3-}, K^+, NH_4^+, Mg^{2+}, and Ca^{2+}) of cAMF-F pellets was considerably higher than that of cAMF pellets. Previously shown in Fig. 5, the AMF mycelia grew faster on cAMF-F pellets than on cAMF pellets. Organic fertilizers are abundant in nutrients. Therefore, the organic fertilizer associated with cAMF-F pellets may stimulate microbial growth in the pellets and soil. Furthermore, in our preliminary experiment involving maize plants grown in pots, we observed that the leaf area and root biomass of plants treated with cAMF-F were 1.4–1.6-fold greater than those of cAMF-treated plants. However, a more comprehensive evaluation of the effects of cAMF-F and cAMF pellets on plants has to be further examined.

3.7. Stability of Stored cAMF and cAMF-F Pellets

The AMF spore germination rate after the 180-day storage period (Fig. 7) was lower than that of the pre-storage cAMF and cAMF-F pellets (Fig. 4). The spore germination rate was higher for cAMF and cAMF-F pellets coated with 1% alginate than for pellets coated with 2 or 3% alginate. It was also higher for pellets coating with one layer of alginate film than for pellets with two layers. Furthermore, the presence of organic fertilizer increased the AMF spore germination rate. After 10 days, the AMF spore germination rates for the cAMF-F pellets with one layer of 1, 2, or 3% alginate film were 96, 84, and 72%, respectively; whereas the pre-storage spore germination rate was 100% for all cAMF and cAMF-F pellets. The growth rates of the resulting mycelia were higher for the cAMF-F pellets than for the cAMF pellets (data not shown), which is consistent with the previous observations in Fig. 5. These results indicate that the added organic fertilizer promotes AMF mycelial growth, and suggest the fertilizer may also increase the shelf life of the AMF spores.

Fungal spores are in general resistant to desiccation and are independent of oxygen; however, AMF are obligate aerobes that requires oxygen for spore germination and hyphal or mycelial growth [36, 37]. One such drawback of alginate gel is the diffusion limitation for oxygen owning to its dense structure [23, 24]. The oxygen supply in alginate was found to decrease with increases in alginate gel thickness and concentration [29]. The storage of cAMF or cAMF-F pellets in plastic bags for 180 days could create a condition of severely deficient supply of oxygen within the pellets resulting in delayed germination of AMF, especially in case of the pellets with 3 % or two layer-alginate coatings as shown in Fig 7. The relatively high coat density from the application of 3% alginate or two layers of alginate film may decrease the oxygen transfer rate, resulting in a lower germination rate after a long storage period. An earlier study revealed that when a formulation consisting of *Phanerochaete chrysosporium* and corn cob grits or sawdust encapsulated with alginate was stored at room temperature for 6 months, 50% of the encapsulated mycelia remained viable [17].

The utility of the plant growth-promoting bacterium *Raoultella planticola* Rs-2 encapsulated within an alginate composite for developing an efficient controlled-release Rs-2 biofertilizer has been previously reported [38]. Encapsulating microbial cells may increase their shelf life and efficacy, while controlling the release of microbes in formulations may enhance the efficacy of these treatments [14]. Appropriate matrices can protect encapsulated cells from the effects of mechanical stress and adverse environmental conditions [39]. *Trichoderma harzianum* and *Glomus* sp. entrapped in alginate beads reportedly had beneficial effects on plants and might be useful for developing viable inoculants adapted to field conditions [22]. The advantages of immobilizing cells on a solid carrier include enhanced regenerative ability, altered mechanical strength, and maintenance of metabolic activity over long periods [39, 40]. In this study, it was demonstrated that the formulation of plant fertilizer pellet composed of biofertilizer and organic fertilizer and coated with thin film of calcium alginate could increase the shelf life of the AMF spores.

3.8. Effects of cAMF and cAMF-F Pellets on Plants

The effects of cAMF and cAMF-F pellets on plants were primarily tested in maize potted plants. The AMF colonization in maize roots was clearly observed from one month old plants inoculated with cAMF and cAMF-F (Fig. 8A and 8B) with the percentages of AMF colonization at 77.00% and 68.33%, respectively (Fig. 8C). AMF colonization in maize roots presents vesicle (v) and intraradical hyphae (h) of the fungi as shown in Fig. 9. No AMF colonization was observed in maize roots of control plants (Con) and plants adding with organic fertilizer (F). The result indicated that the mycelia observed from germination of fungal spores on cAMF and cAMF-F pellets should belong to mycorrhizal fungi and mycorrhiza was able to invest into root tissues in term of symbiosis as hyphae, vesicles and arbuscules.

Phosphorus (P) fertilizer has been reported its inhibition effect to mycorrhizal colonization and growth. From a previous study of P effect on maize inoculated with *G. etunicatum* by watering the plants with modified Hoagland solution containing 5 mM phosphate (HP) and 50 μM phosphate (LP), respectively, it had been reported that the HP condition was associated with significantly lower levels of mycorrhizal colonization, smaller arbuscules and slower arbuscular development than the LP condition [41]. In the present study, the total concentration of P in cAMF-F pellet was higher than cAMF pellet. However, the P content in form of phosphate in cAMF-F pellet was considered very low (less than 0.25% of the total weight of cAMF-F) as compared to those of inorganic P fertilizers. It was shown that under the slow release of P from the cAMF-F pellets, no significant effect of P release on the percentage of AMF colonization in the root of maize was observed.

For the further study of the effect of cAMF and cAMF-F pellets on drought stress tolerance, the one month old plants of control plants (Con) and the one month old plants inoculated with fertilizer (F), cAMF pellets and cAMF-F pellets were evaluated for a limitation of water supply by non-irrigation for 2 weeks. The SWC percentages in the irrigated condition and in water deficit (non-irrigation for 2 weeks) were 55% and 34%, respectively. As shown in Table 2, significant reductions of water content in maize shoots of control and F plants at 4.10 % and 2.28%, respectively were detected. On the other hand, those of cAMF and cAMF-F plants showed only slight changes of water content in the shoots under the water deficit treatment for 2 weeks. The cAMF-F plant under the water deficit was found to maintain with a slight increase of water content in the shoots. Previously, maize plants (*Zea mays L.*) treated by inoculation with AMF were recorded a significant increase in plant growth parameters and biochemical compounds [plant height, stem length, root length, plant fresh weight, shoot dry weight, root dry weight, plant chlorophyll content and phosphorus (P) uptake] comparing with untreated plants in both normal irrigation and drought stress [42]. AMF had shown to enhance drought tolerance in various plants through physiological mechanisms in nutrient uptake and biochemical mechanisms regarding hormones, osmotic adjustment, and antioxidant systems [43, 44].

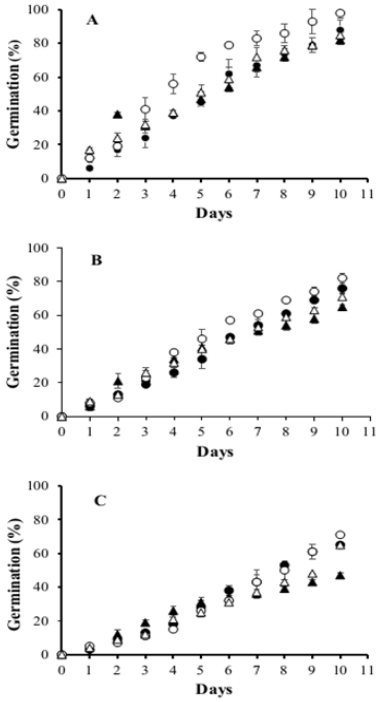

Fig. 7. Germination percentages of AMF mycelium from cAMF and cAMF-F pellets after the 6 month-storage by coating with different concentrations of alginate at 1% (A), 2% (B) and 3% (C): cAMF,1L (●); cAMF, 2L (▲); cAMF-F,1L (○); cAMF-F,2L (Δ) (mean±SE, n=50).

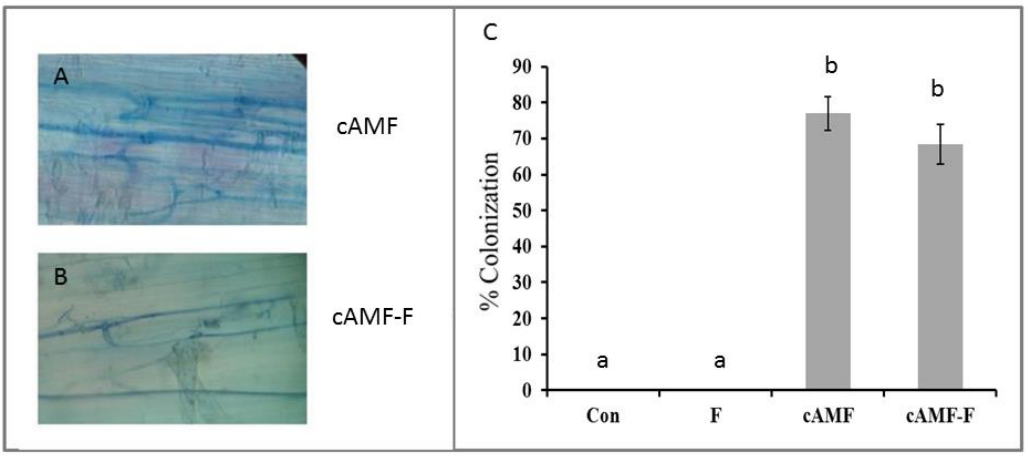

Fig. 8. AMF colonization in maize roots of one month old plants inoculated with cAMF (A) and cAMF-F (B) and colonization (%) of AMF in maize root (C) of control plants (Con) and plants inoculated with organic fertilizer (F), cAMF pellets and cAMF pellets (mean±SE, n=5, Tukey ($p \leq 0.05$)).

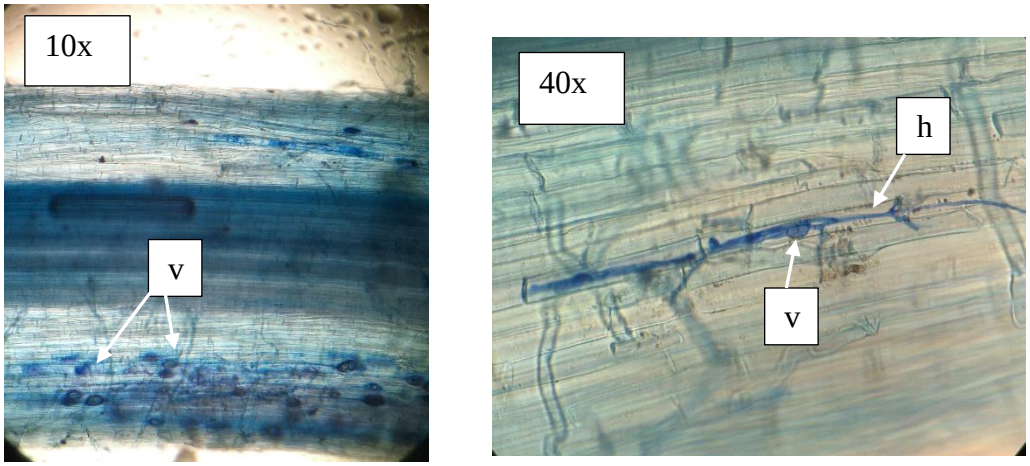

Fig. 9. Enlarged view of AMF colonization in cAMF-F maize roots to present vesicle (v) and intraradical hyphae (h) of the fungi.

Table 2. Reductions of water content (%) in maize shoots of control plants (Con), and plants inoculated with organic fertilizer (F), cAMF pellets and cAMF-F pellets between irrigated and water deficit conditions (mean±SE, n=4), Tukey's HSD ($p \leq 0.05$)).

Reductions of water content (%)			
Con	F	cAMF	cAMF-F
4.10 ± 0.91a	2.28± 0.70ab	0.41±1.28ab	-0.38±0.63b

4. Conclusions

Granular cAMF and cAMF-F pellets encapsulated by thin calcium alginate films have been successfully developed in this study. The hardness of the pellets could be increased from 7–8 N to about 80 N by increasing the alginate concentration in the coating film from 1 to 3%. We observed that 2% sodium alginate was the ideal formulation regarding the fungal spore germination rate and mechanical properties of the cAMF and cAMF-F pellets. It was found that the presence of organic fertilizer in the cAMF-F pellets significantly promoted the growth of AMF mycelia. In soil, the initial decomposition rate of cAMF-F pellets was higher than that of cAMF pellets. However, both types of pellets were fully degraded within 30 days. The supplement of organic fertilizer in biofertilizer in form of cAMF-F can help to promote hyphal growth of AMF and

increase the shelf life of the AMF stock culture, therefore the cAMF-F could have more potential to promote growth, productivity and resistance to drought stress of plants. In form of cAMF-F pellet, the AMF spores remain viable over long storage periods (e.g., 6 months). Additionally, these pellets are also easy to handle and apply. It was demonstrated that AMF and AMF-F could promote colonization and provided resistance to drought stress in maize potted plants. Furthermore, these pellets may be relevant for soil and nutrient management practices during the production of other crops under organic conditions.

Acknowledgements

The authors are grateful for the financial support from the Thailand Research Fund; contract Grant No.SRI5920201.

References

[1] T. Thaiturapaisan. (2016). *Thailand's Drought Crisis 2016: Understanding It without the Panic* [Online]. Available: https://www.scbeic.com/en/detail/product/ 2127/ [Accessed: 29 May 2017]
[2] M. He and F. A. Dijkstra, "Drought effect on plant nitrogen and phosphorus: A meta-analysis," *New Phytol.*, vol. 204, pp. 924-931, 2014.
[3] M. Brundrett, "Mycorrhizas in natural ecosystem," *Adv. Ecol. Res.*, vol. 21, pp. 171-313, 2009.
[4] M. J. Harrison, G. R. Dewbre, and J. Liu, "A phosphate transporter from Medicago truncatula involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi," *Plant Cell*, vol. 14, no. 10, pp. 2413–2429, 2002.
[5] J. Meesumlee and T. Intala, "Effect of phosphorus in arbuscular mycorrhizal fungi dependency for growth and yield of chili," *Khon Kaen Agr. J.*, vol. 43, no. 2, pp. 343-352, 2015.
[6] K. Jing, P. Zongping, D. Min, S. Gan, and Z. Xin, "Effects of arbuscular mycorrhizal fungi on the drought resistance of the mining area repair plant Sainfoin," *Int. J. Min. Sci. Technol.*, vol. 24, pp. 485-268, 2014.
[7] P. Poomipan, "Roles of arbuscular mycorrhizal fungi on plant, soil and environment," *Thai J. Sci. Technol.*, vol. 2, pp. 91-101, 2013.
[8] Q. S. Wu and Y. N. Zou, "Mycorrhiza has a direct effect on reactive oxygen metabolism of drought-stressed citrus," *Plant Soil Environ.*, vol. 55, no. 10, pp. 436-442, 2009.
[9] E. A. Gomes, C. A. Oliveira, U. G. Lana, R. W. Noda, I. E. Marriel, and F. A. de Souza, "Arbuscular mycorrhizal fungal communities in the roots of maize lines contrasting for Al tolerance grown in limed and non-limed Brazilian Oxisoil," *J. Microbiol. Biotechnol.*, vol. 25, pp. 978-987, 2015.
[10] Y. Wei, Z. Chen, F. Wu, J. Li, Y. ShangGuan, F. Li, Q.R. Zeng, and H. Hou, "Diversity of arbuscular mycorrhizal fungi associated with a Sb accumulator plant, Ramie (Boehmeria nivea), in an active Sb mining," *J. Microbiol. Biotechnol.*, vol. 25, no. 8, pp. 1205-1215, 2015.
[11] L. Herrmann and D. Lesueur, "Challenges of formulation and quality of biofertilizers for successful inoculation," *Appl. Microbiol. Biotechnol.*, vol. 97, no. 20, pp. 8859-8873, 2013.
[12] A. Jarosiewicz and M. Tomaszewska, "Controlled release NPK fertilizer encapsulate by polymetric membranes," *J. Agric. Food Chem.*, vol. 51, no. 2, pp. 413-417, 2003.
[13] X. Han, S. Chen, and X. Hu, "Controlled-release fertilizer encapsulated by starch/polyvinyl alcohol coating," *Desalination*, vol. 240, pp.21-26, 2009.
[14] R. P. John, R. D. Tyagi, S. K. Brar, R. Y. Surampalli, and D. Prévost, "Bio-encapsulation of microbial cells for targeted agricultural delivery," *Crit. Rev. Biotechnol.*, vol. 31, no. 3, pp. 211-226, 2011.
[15] M. Tomaszewska and A. Jarosiewicz, "Encapsulation of mineral fertilizer by polysulfone using a spraying method," *Desalination*, vol. 198, pp. 346-352, 2006.
[16] V. Nedovic, A. Kalusevic, V. Manojlovic, S. Levic, and B. Bugarski, "An overview of encapsulation technologies for food applications," *Procedia Food Sci.*, vol. 1, pp. 1806-1815, 2011.
[17] J. W. Bennett, A. J. Turner, A. K. Lomis, and W. J. Connick, "Comparison of alginate and Pesta for formation of Phanerochaete chrysosporium," *Biotecnol. Tech.*, vol. 10, pp. 7-12, 1996.
[18] K. Tanaka, K. Ohta, P. R. Haddad, J. S. Fritz, K. P. Lee, K. Hasebe, A. Ieuji, and A. Miyanaga, "Acid-rain monitoring in East Asia with a portable-type ion-exclusion-cation-exchange chromatographic analyzer," *J. Chromatog.*, vol. 850, pp. 311–317, 1999.

[19] G. S. Hossain, R. Waditee, T. Hibino, Y. Tanaka, and T. Takabe, "Root specific expression of Na^+/H^+ antiporter gene from Synechocystis sp. PCC6803 confers salt tolerance of tobacco plant," *Plant Biotechnol.*, vol. 23, pp. 275–281, 2006.
[20] S. Yooyongwech, T. Samphumphuang, R. Tisarum, C. Theerawitaya, and S. Cha-um, "Arbuscular mycorrhizal fungi (AMF) improved water deficit tolerance in two different sweet potato genotypes involves osmotic adjustments via soluble sugar and free proline," *Sci Hort.*, vol. 198, pp.107–117, 2016.
[21] S. Yooyongwech, C. Theerawitaya, T. Samphumphuang, and S. Cha-um, "Water-deficit tolerant identification in sweet potato genotypes (*Ipomoea batatas* (L.) Lam.) in vegetative developmental stage using multivariate physiological indices," *Sci Hort.*, vol. 162, pp. 242–251, 2013.
[22] N. De Jaeger, I. E. de la Providencia, H. Rouhier, and S. Declerck, "Co-entrapment of Trichoderma harzianum and *Glomus* sp. within alginate beads: Impact on the arbuscular mycorrhizal fungi life cycle," *J. Appl. Microbiol.*, vol. 111, pp. 125-135, 2011.
[23] Y. Kourkoutas, A. Bekatorou, I. M. Banat, R. Marchant, and A. A. Koutinas, "Immobilization technologies and support materials suitable in alcohol beverages production: A review," *Food Microbiol.*, vol. 21, pp. 377-397, 2004.
[24] P. J. Verbelen, D. P. de Schutter, F. Delvaux, K. J. Verstrepen, and F. R. Delvaux, "Immobilized yeast cell systems for continuous fermentation applications," *Biotechnol Lett.*, vol. 28, pp. 1515-1525, 2006.
[25] J. Mongkolkajit, J. Pullsirisombat, S. Limtong, and M. Phisalaphong, "Alumina-doped alginate gel as a cell carrier for ethanol production in a packed-bed bioreactor," *Biotechnol Bioprocess Eng.*, vol. 16, pp. 505-512, 2011.
[26] F. O. Sonmez and A. Demir, "Analytical relations between hardness and strain for cold formed parts," *J. Mater. Process Tech.*, vol. 186, pp. 163–173, 2007.
[27] Y. He, Z. Wu, L. Tu, Y. Han, G. Zhang, and C. Li, "Encapsulation and characterization of slow-release microbial fertilizer from the composites of bentonite and alginate," *Appl. Clay Sci.*, vol. 109-110, pp. 68-75, 2015.
[28] N. Kanjanamosit, C. Muangnapoh, and M. Phisalaphong, "Biosynthesis and characterization of bacteria cellulose–alginate film," *J. Appl. Polym. Sci.*, vol. 115, pp. 1581–1588, 2010.
[29] J. C. T. Dias, R. P. Rezende, V. and R. Linardi, "Effects of immobilization in Ba-alginate on nitrile-dependent oxygen uptake rates of Candida guilliermondii," *Braz. J. Microbiol.*, vol. 32, pp. 221-224, 2001.
[30] U. Hildebrandt, K. Janetta, and H. Bothe, "Towards growth of arbuscular mycorrhizal fungi independent of a plant host," *Appl. Environ. Microbiol.*, vol. 68, pp. 1919–1924, 2002.
[31] K. Kuwada, M. Kuramoto, M. Utamura, I. Matsushita, and T. Ishii, "Isolation and structural elucidation of a growth stimulant for arbuscular mycorrhizal fungus from Laminaria japonica Areschoug," *J. Appl. Phycol.*, vol. 18, pp. 795–800, 2006.
[32] S. Mandal, S. S. Kumar, B. Krishnamoorthy, and S. K. Basu, "Development and evaluation of calcium alginate beads prepared by sequential and simultaneous methods," *Braz. J. Pharm. Sci.*, vol. 46, pp. 785-793, 2010.
[33] R. Liang, M. Liu, and L. Wu, "Controlled release NPK compound fertilizer with the function of water retention," *Reac. Func. Poly.*, vol. 67, pp. 769-779, 2007.
[34] Z. Liu, Y. Jiao, Y. Wang, C. Zhou, and Z. Zhang, "Polysaccharides-based nanoparticles as drug delivery systems," *Adv. Drug. Deliv. Rev.*, vol. 60, no. 15, pp. 1650–1662, 2008.
[35] B. Singh, D. K. Sharma, R. Kumar, and A. Gupta, "Controlled release of the fungicide thiram from starch–alginate–clay based formulation," *Appl. Clay Sci.*, vol. 45, pp. 76-82, 2009.
[36] M. L. Fazenda, L. M. Harvey, and B. McNeil, "Effects of dissolved oxygen on fungal morphology and process rheology during fed-batch processing of Ganoderma lucidum," *J. Microbiol. Biotechnol.*, vol. 20, no. 4, pp. 844–851, 2010.
[37] W. K. Cornwell, B. L. Bedford, and C. T. Chapin, "Occurrence of arbuscular mycorrhizal fungi in a phosphorus-poor wetland and mycorrhizal response to phosphorus fertilization," *Am. J. Bot.*, vol. 88, no. 10, pp.1824–1829, 2001.
[38] Z. Wu, L. Guo, S. Qin, and C. Li, "Encapsulation of R. planticola Rs-2 from alginate-starch-bentonite and its controlled released and swelling behavior under simulated soil conditions," *J. Ind. Microbial. Biotechnol.*, vol. 39, pp. 317–327, 2012.
[39] M. Phisalaphong, R. Budiraharjo, P. Bangrak, J. Mongkolkajit, and S. Limtong, "Alginate-loofa as carrier matrix for ethanol production," *J. Biosci. Bioeng.*, vol. 104, no. 3, pp. 214–217, 2007.
[40] M. B. Cassidy, H. Lee, and J. T. Trevors, "Environmental applications of immobilized microbial cells: A review," *J. Ind. Microbiol.*, vol. 16, no. 2, pp. 79–101, 1996.

[41] F. Liu, Y. Xu, G. Han, W. Wang, X. Li, and B. Cheng, "Identification and functional characterization of a maize phosphate transporter induced by mycorrhiza formation," *Plant Cell Physiol.*, vol. 59, pp. 1683–1694, 2018.
[42] T. S. Abdelmoneim, A. A. Tarek, O. A. Almaghrabi, and A. Ismail, "Increasing plant tolerance to drought stress by inoculation with arbuscular mycorrhizal fungi," *Life. Sci. J.*, vol. 10, no.4, pp. 3273-3280, 2013.
[43] M. J. Pozo, J. A. López-Ráez, C. Azcón-Aguilar, and J. M. García-Garrido, "Phytohormones as integrators of environmental signals in the regulation of mycorrhizal symbioses," *New Phytol.*, vol. 205, pp. 1431–1436, 2015.
[44] Q. S. Wu and Y. N. Zou, "Arbuscular mycorrhizas fungi and tolerance of drought stress in plants," in *Arbuscular Mycorrhizas and Stress Tolerance of Plants*, 1st ed. Singapore: Springer Nature Singapore Pte Ltd, 2017, ch. 2, pp. 25–41.