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Effectiveness of Three Forms of *Frankia* Inoculants as Influenced by Storage Conditions

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THE INFLUENCE of different storage length and storage temperatures on growth, amount of viable cells and spore production of *Frankia* cultures was examined *in vitro* at different intervals. Results revealed that the maximal total protein content and the maximal viability of cells were observed 10 days after initiation of the culture. Gradual decreases either in growth or cells viability were observed at all tested storage length at 28°C or 6°C. Extension of storage period or temperature positively affected spore production. The spore formation was quite intensive in *Frankia* cultures after 10 days at 28°C.

The effectiveness of three types of *Frankia* inoculums as influenced by storage conditions on the performance of *Casuarina glauca* seedlings was studied in a pot experiment carried out outdoors under a net.The experimental design was a 3:2:4 factorials comprising the following treatments: 1) inoculum types: spore enriched *Frankia* culture (SEC); alginate entrapped inoculum (AEI); alginate entrapped inoculum + kaolinite (AEIK), 2) storage temperature: 6°C; 28°C, 3) storage length: 1 month; 3 months; 6 months; 12 months.

Results showed that the above-mentioned interactions revealed a highly significant effect ($P<0.01$) on plant growth and N_2-fixation. Inoculant types exhibited marked significant differences in their effects and the AEIK inoculant was the most effective form. Plant, nodule dry weights and fixed nitrogen increased significantly with AEIK inoculant stored either at 28C° or 6C°. The inoculant types and storage length interaction also revealed significant increase in plant and nodule dry weights, nodule efficiency and N_2-fixation with AEIK inoculant in all storage length. One the other hand, plant height and calculated specific acetylene reduction activity (SARA) were not significant for all studied treatments. The interactions of the three studied factors showed a highly significant effect ($P<0.01$) on plant dry weight and N_2-fixation. While the maximum amounts of plant and nodule dry weights with AEIK inoculant were obtained after 3 and 6 months of storage at 28C°, the maximum amounts of nodule efficiency and N_2-fixed with the same inoculant were obtained after the first and the third months, respectively.

Keywords: Storage length, Storage temperature, *Frankia* inoculant, Growth, Nitrogen fixation, *Casuarina glauca* .

The *Casuarina-Frankia* symbiosis is of particular importance for arid or semi-arid land reclamation (Diem and Dommergues 1990; Girgis *et al.*, 1992), to get improved and reliable nodulation and to ensure increased plant productivity. The use of pure cultures of *Frankia* with high cell viability allows selecting a better combination of *Casuarina-Frankia* for maximum nitrogen fixation (Girgis *et al.*, 1990 and 2002). Inoculation of seedlings with effective strains of *Frankia* offers several potential advantages, such as, (a) better survival rate of transplanted seedlings; (b) faster start growing tree with more capability of competition with weeds; (c) little or no dependence on N-fertilization and (d) potential production of biomass yield/hectare in shorter rotation time (Steele *et al.*, 1989).

Although liquid cultures of *Frankia* have worked successfully, there are obvious logistical problems in applying this method in boarder scale. Large volume of liquid inocula is required to inoculate a reasonable size forest nursery but transport of these liquid cultures from the laboratory to isolated nurseries is impractical.

During the last decade, several experimental formulations based on polymers have been evaluated (Bashan, 1986 and 1998; Sayed *et al.*, 2002). These polymers were demonstrated as potential bacterial carrier (Jung *et al.*, 1982) offering substantial practical advantages over peat as a traditional carrier (Amiet-Charpentier *et al.*, 1998 and 1999). They can be stored dry at ambient temperatures for prolonged periods, offer consistent batch quality and a better-defined conditions for the bacteria, and can be manipulated easily according to the needs of specific bacteria or the crop (Bashan *et al.*, 2002). The survival of the entrapped *Frankia* in alginate polymer could be enhanced through some additives such as clay (Kaolinite) which is desirable for producing effective inoculant (Girgis *et al.*, 1991; Girgis, 1993; Girgis and Mostafa, 2002).

The objective of this investigation was to study the influence of different storage length (1, 3, 6 and 12 months) and two storage temperatures (6°C and 28°C) of three types of *Frankia* inoculants on their performance when applied for *Casuarina glauca* seedlings.

Material and Methods

The experiments were conducted in Wadi-el-Natroon nursery belonging to the Central Administration for the Afforestation, Egyptian Ministry of Agriculture, Giza, Egypt.

Raising of casuarina seedlings

Seeds of local *Casuarina glauca* were surface sterilized by immersing them for 20min in 5% (w/v) calcium hypochlorite, and then washed with sterile distilled water (Girgis *et al.*, 1992). Sterilized seeds were germinated in a sterile bed of sand and peat mixture (2:1 w/w). Five weeks after germination, two

seedlings averaging 4cm in shoot height were transferred into 15cm-diameter pot containing sterilized non-saline non-alkaline sandy soil (86% sand, 3% silt and 9% clay; pH 8.07; E.C 1.73 mmhos/cm^2; % 0.04organic carbon; % 0.01 total nitrogen; 2ppm total phosphorus and % 2.87 $CaCO_3$).

Seedlings were fed for 4 weeks (twice a week) with ¼ strength nutrient solution (Hoagland and Arnon, 1950) containing NH_4^+ and then for 3 weeks with NH_4^+ - free nutrient solution under net house conditions with mean day and night temperatures of 32°C and 22 °C, respectively.

Propagation of Frankia

Frankia strain Sa1 (Catalogue no. UBF 020803) previously isolated from *C. glauca* by Girgis *et al.,* (2002) was stored in Qumod medium (Lalonde and Calvert, 1979) and sub-cultured every three months. *Frankia* cultures were propagated in BAP-PCM medium as recommended by Girgis and Schwencke (1993) under stirred conditions to obtained exponentially growing hyphae. The developed active hyphae were collected after 5 days growth by centrifugation at 5000 rpm for 10 min, washed, homogenized and re-suspended in sterile distilled water. The inoculum was diluted to contain 2µg of mycelial protein ml^{-1} and 4nmol INTF/ml (v/v) to be used as a standard inoculum. The growth and the level of cells viability in *Frankia* culture were determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard protein and 2-(*p*-iodophenyl-3-(*p*-nitrophenyl)-5-(phenyl-tetrazolim chloride) reduction activity technique (IRA) as described by Prin *et al.,* (1990), respectively. The IRA technique reflects the amount of viable cells of *Frankia* in the culture. Cultures grown at 28°C were magnetically stirred at 150 rpm with a cross-type magnetic bar as recommended by Girgis (2002).

Preparation of Frankia inoculants

Three types of *Frankia* inocula were prepared from exponential growing culture. To obtain spore-enriched cultures (SEC) of *Frankia*, the Sa1 strain was inoculated with the standard inoculum in BAP-PCM nitrogen-free medium, without PC but supplemented with 0.1% purified agar (Oxoid, P/L) as described by Girgis (2001). SEC were entrapped in alginate (Na-alginate *e.g.,* Alginate s170 manufactured by SATIA, 15 avenue de Eylau, 75116 Paris, France) according to the method of Diem and Dommergues (1990). Cultures were amended with 5% Na-alginate (AEI) or 5% Na-alginate plus 10% kaolinite (AEIK) and slurry was passed drop-wise from a burette into about 300 ml of 0.1 M $CaCl_2$ solution under magnetic stirring. The formed beads were kept in 0.1M $CaCl_2$ solution for 15 min, harvested and washed several times with sterile water, dried in a laminar flow hood and stored in sterile closed containers.

Experimental

Evaluation of IRA and effectiveness of Frankia inoculants

The three types of inocula SEFC, AEI and AEIK were tested after 1, 3, 6, and 12 months storage length either in room temperature (28°C ±2) or in refrigerator

(6°C ±2) under static conditions. The cellular protein and IRA of *Frankia* culture (SEC) were periodically determined as described before. Five weeks-old *Casuarina glauca* seedlings were grouped to be inoculated with *Frankia* inoculant representing the above-mentioned treatments.

The effectiveness of three types of *Frankia* inoculums as influenced by storage conditions on the performance of *C. glauca* seedlings was studied in a pot experiment carried out outdoors under a net. The experimental design was a 3: 2: 4 factorials comprising the following treatments:
- inoculum types: SEC, AEI and AEIK
- storage temperature: 6°C and 28°C
- storage length: 1, 3, 6 and 12 months.

Frankia inoculants were applied at a rate of 50µg total protein content/seedling for SEC inoculant or 40 and 140mg of AEI and AEIK polymeric inoculants/ seedling, respectively (Girgis *et al.*, 1992; Girgis, 1993; Girgis and Said, 2002). The polymer-entrapped microbial inoculant was applied to the seedlings as a pseudo-solution obtained by immersing the dried beads into a phosphate buffer solution (0.03M KH_2PO_4 plus 0.17M K_2PO_4; pH 7.4 for 3hr) as recommended by Girgis (1993). Un-inoculated (control) seedlings received an amount of buffer solution equivalent to that applied in other inoculated treatments. Ten replicates were made for each treatment.

Effect of storage length and storage temperature on growth, IRA and spore production of Frankia in vitro

Growth pattern and IRA of *Frankia* cultures were carried out in 250ml vials containing 100ml of BAP-PCM nitrogen-free medium, without egg yolk phosphetidyl coline mixture (PC) but supplemented with 0.1% (w/v) purified agar. Cultures were started with 2µg of mycelial protein ml^{-1} from an exponentially growing culture as described by Girgis and Schwencke (1993). Cultures were grown at 28°C or 6°C under static conditions. Cultures were harvested by centrifugation at 5000 rpm and the total cellular protein, IRA as well as the number of *Frankia* spores was determined at different intervals (10 days, 1, 3, 6 and 12 months). The spore density was evaluated by direct microscopic counting using a haemocytometer (Girgis 2001).

Parameters measured

The seedlings were examined six months after inoculation to record plant height, total dry weight of the plant, the number and dry weight of nodules and total nitrogen content determined by the Kjeldahel method (Bremner and Mulvaney, 1982). Nitrogenase activity of nodulated plants was determined using acetylene reduction assay (Hardy *et al.*, 1968). A measurable and reproducible chromato-graphic signal with whole nodulated plants was obtained after 3 hr of incubation with acetylene at 28C°. Assays were performed using whole nodulated plants under near-identical conditions to avoid unwanted variables as discussed by Huss-Danell (1990) and Vessey (1994). The specific acetylene

reduction activity (SARA) was also expressed as nmol $C_2H_4g^{-1}$nodule dry weight h^{-1}. Nitrogen fixation was estimated using the total-N difference method (difference in N content between inoculated plants and uninoculated control plants) as described by Sougoufara *et al.* (1989) and Galiana *et al.* (1990). Nodules efficiency was expressed by the ratio of N_2-fixed to nodules dry weight. This characteristic is closely related to the nodulation efficiency, a concept proposed by Döberiner *et al.* (1970).

Data analysis

The experimental design was apparently a factorial device comprising three treatments at different levels. Inoculant types (un-inoculated, SEC, AEI and AEIK); Storage length (1, 3, 6, and 12 months) and Storage temperature (28°C and 6°C). Therefore, data was subjected to variance analysis to test the effect of inoculant type, storage duration and temperature storage and the different interactions. Data were statistically analyzed according to Snedecor and Cochran (1989), using Costat software (1985).

Results

Growth rate, IRA and spore production by Frankia

The cellular protein and the cells viability measured by IRA of spore-enriched *Frankia* cultures (SEC) grown under static conditions showed an exponential growth till the 10^{th} day of incubation (Fig.1a). The highest amount of protein with a maximal increase of cells viability were 42.3µg/ml and 108.35µmol INTF/ml respectively. This means that the activity precedes the growth. On the other hand, a gradual decrease either in total protein content or IRA was observed after storage length of 1, 3, 6, and 12 months either in room temperature (28°C) or in refrigerator (6°C) under static conditions.

Data presented in Fig.1b clearly showed that both storage period and storage temperature positively affected spore production. The spore formation was quite insensitive in *Frankia* cultures after 10 days at 28°C. However, the highest densities of spores were 40.8 $x10^5$ and 23.9 $x10^5$ spore/ml, after 6 and 12 months of storage at 28°C and 6°C, respectively.

Evaluation of the effects of Frankia inoculant type, storage length and storage temperature on the performance of Casuarina glauca seedlings

Results recorded in Table 1 revealed that the inoculant type significantly affected their performances. Comparing the effect of inoculants type, results in Table 1 showed that AEIK inoculant was the most effective form. Regarding the storage temperature of the inoculant, a highly significant effect ($P<0.01$) for all studied parameters at 28C° was obtained. Concerning the effect of storage length on the effectiveness of *Frankia* inoculant, data in Table 1 also showed that, regardless the recorded parameter, the storage length was highly significant ($P<0.01$) with both growth and N_2-fixation parameters.

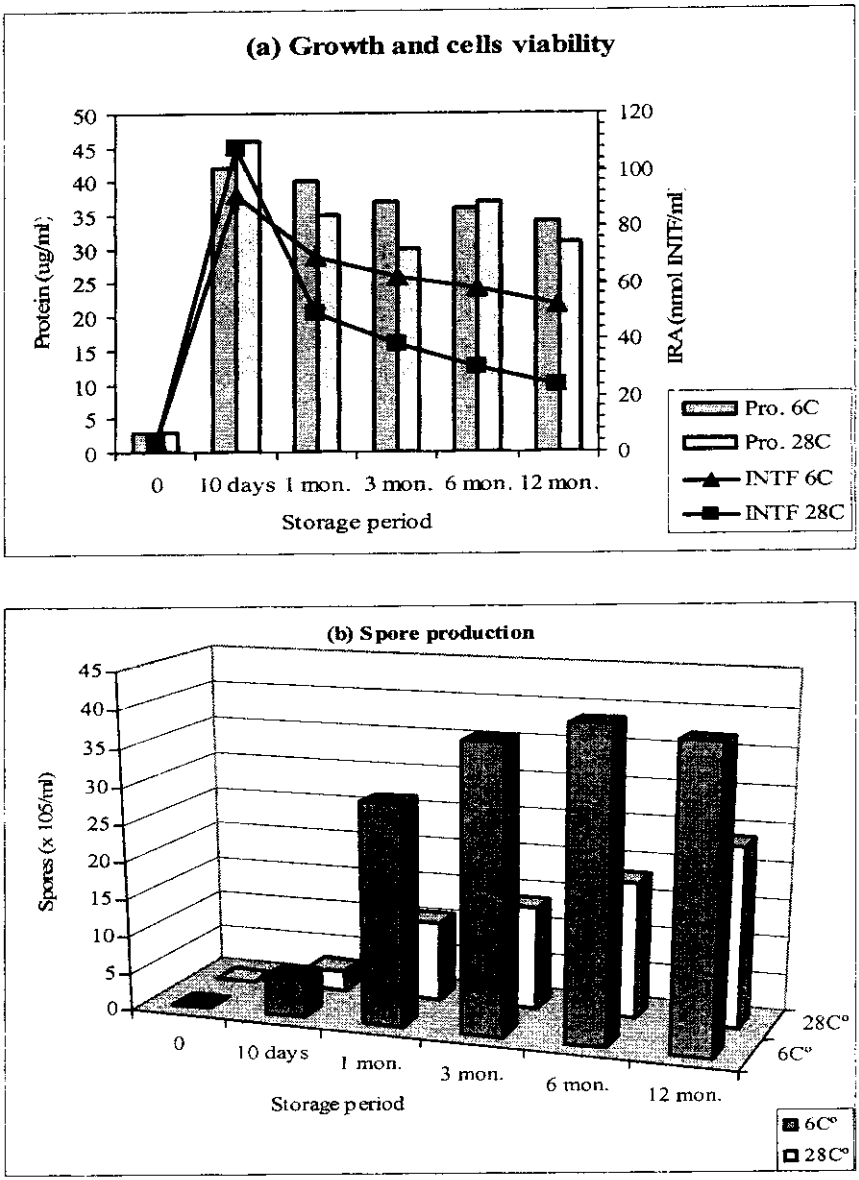

Fig. 1. Growth and cell viability (a) and Spore production (b) of *Frankia* Sa1 grown under static conditions in BAP-PCM free nitrogen medium supplemented with 0.1% agar at different intervals of storage at 6°C or 28°C .

TABLE 1. Effect of inoculum type, storage temperature and storage length on the different parameters related to growth, nodulation and nitrogen fixation of *Casuarina glauca* grown under net house conditions .

Treatment	Plant height (cm/plant)	Plant dry weight (mg/plant)	Nodule number (per plant)	Nodule dry weight (mg/plant)	Total nitrogen content (mg/plant)	Fixed nitrogen (mg/plant)	N_2-ase activity (nmol C_2H_4/ plant/h)	SARA (nmol C_2H_4/g nodule dry weight/h)	Nodules efficiency (N_2-fixation/ nodule dry weight)
Inoculum type: (1)									
Control	30.1 a	2494.4 d	0 d	0 c	284.4 c	0 c	0 c	0 a	0 c
Spore-enriched *Frankia* cultures (SEC)	39.1 a	2916.5 c	9.1 c	675.5 b	477.2 b	192.8	3163.7 b	19.6 a	0.4 b
Alginate (AEI)	45.5 a	3602.5 b	10.4 b	652.2 b	474.1 b	186.5 b	2937.5 b	5.2 a	0.4 b
Alginate+ kaolin. (AEIK)	41.7 a	4256.2 a	14.4 a	838.2 a	646.2 a	361.8 a	3718.5 a	4.8 a	0.5 a
Storage temperature (2)									
6C°	41.2 a	3233.0 b	6.1 b	467.0 b	438.0 b	150.4 b	2205.6 b	4.2 a	0.3 b
28C°	37.0 a	3401.9 a	10.8 a	615.9 a	502.9 a	220.2 a	2704.2 a	10.6 a	0.4 a
Storage length (3):									
1 month	45.3 a	3155.0 b	3.4 c	123.5 b	404.4 d	105.8 c	1061.6 c	6.7 a	0.6a
3 months	37.1 a	3425.6 a	8.9 b	637.1 a	544.4 a	252.4 a	2638.8 b	16.4 a	0.3 b
6 months	38.4 a	3465.6 a	11.2 a	677.8 a	491.7 b	233.3 a	3107.0 a	3.4 a	0.2 b
12 months	35.7 a	3223.5 b	10.3 a	727.4 a	441.5 c	149.7 b	3012.3 a	3.2 a	0.1 c
Level of significance: S***P<0.01, NS=not significant.									
Inoculum type effect:	NS	S***	S***	S***	S***	S***	S***	NS	S***
Storage temperature:	NS	S**	S***	S***	S***	S***	S***	NS	S**
Storage length:	NS	S***	S***	S***	S***	S***	S***	NS	S***
Interaction:									
Inoculum type x storage temp.:	NS	s**	S***	S***	S***	S***	S***	NS	NS
Inoculum type x storage length:	NS	s**	S***	S***	S***	S***	S***	NS	S***
Inoculum type x storage temp. x Storage x length:	NS	s**	S**	S***	S***	S***	NS	NS	S***

The variance analysis was carried out on log transformed data. Numbers followed by the same letter in a given column do not differ significantly at P<0.01.
(1) Averaged data from storage length and inoculum type (2) Averaged data from temperature and inoculum type (3) Averaged data from temperature and storage length.

Plant dry weight and N_2-fixation activity significantly increased after 3 and 6 month of inoculant storage, whereas the nodulation of *Casuarina glauca* seedlings and N_2-ase activity significantly increased after 6 and 12 months of inoculant storage. No significant differences were found in nodule dry weight after 3, 6 and 12 months of inocula storage; however, the three storage length together showed an increase in nodule dry weight which were higher than the recorded obtained after the month of storage. On the other hand, within the same period, highly significant nodule efficiency (0.62 N_2-fixation/nod.dry weights) was observed compared to the other storage length.

Inoculant type/storage temperature interaction

Regarding the effects of inoculant type and storage temperature interaction, results presented in Fig. 2 showed that both plant growth and N_2-fixation were both significantly affected ($P<0.01$) and that the plant dry weight increased with AEIK inoculant stored either at 28C° or at 6C°. The relative percentage increases (R I %) of plant dry weight with AEIK inoculant were higher by 72% and 68% than un-inoculated treatment (control) at 28C° and 6C°, respectively. Nodule dry weight was significantly high with AEIK inoculant storage at 6C° and 28C°. The increases in nodule dry weight were higher by 16.5% and 50% at 28C° than the SEC inocula. Significant increase in N_2-fixation was observed with AEIK inoculant stored either at 6C° or 28C° compared to other inoculant. Figure 2 showed that N_2-fixation decreased when SEC inoculant was stored at 6C° than at 28C°. Thus, the level of increases in N_2-fixation with AEIK inoculant was higher by 191% and 45% than SEFC inocula at 6C° and 28C°, respectively. Moreover, AEIK inoculant showed high nodule efficiency (0.5 and 0.6 N_2-fixation/nod. dry weight) with storage at 6C° and 28C°. The levels of N_2-fixation with AEIK inoculant were higher by 25%, 67% and 50% than AEI and SEC inocula at 6C° and 28C°, respectively.

Inoculant type/storage length interaction

Concerning the combination of inoculant types and storage length, results in Fig. 3 showed a significant influence ($P<0.01$) on both plant growth and N_2-fixation. Significant increase in plant dry weight was observed with AEIK inoculant in all storage length. The highest amounts of plant dry weight were 4.6, 4.4, 4.9 (g/plant) after 3, 6 and 12 months of storage length of AEIK inoculant, respectively. The increases in plant dry weight when using AEIK inoculant were higher by 84%, 76% and 60% than un-inoculated treatment (control). Nodule dry weight significantly increased with AEIK inoculant, and the maximum recorded was 1237 mg/plant after 6 month of storage. The levels of increases in nodule dry weight using AEIK inoculant were higher by 40%, 33%, 28% than SEC and AEI inoculants after 3, 6 and 12 months of storage, respectively. The same trend was also observed with both N_2-fixation and nodule efficiency, as AEIK was the most effective type of inocula. The highest amounts of N_2-fixation when using AEIK inoculant (496, 429, 321 N_2-fixation/nod.dry weight) were detected after 3, 6 and 12 months, respectively.

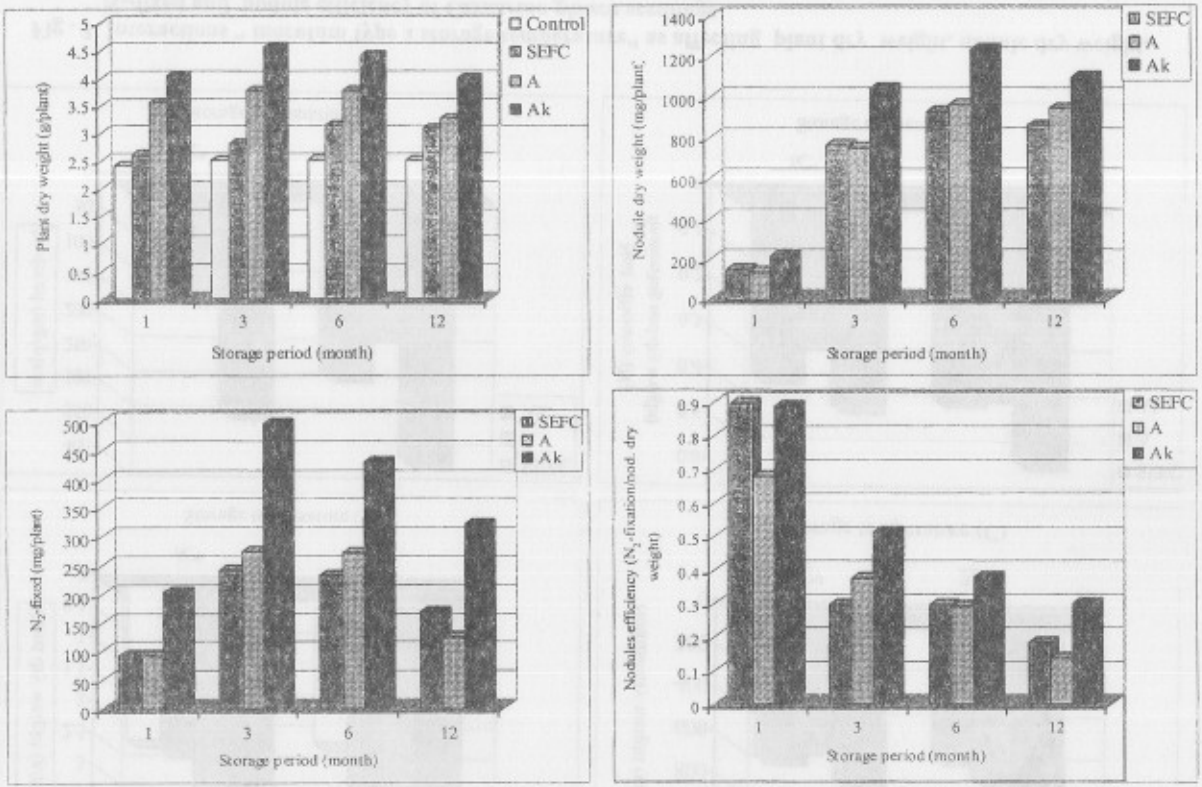

Fig . 2. Interactions " inoculum type x storage length " as affecting plant dry weight, nodule dry weight, N_2-fixed and nodule efficiency of *Casuarina glauca* seedlings.

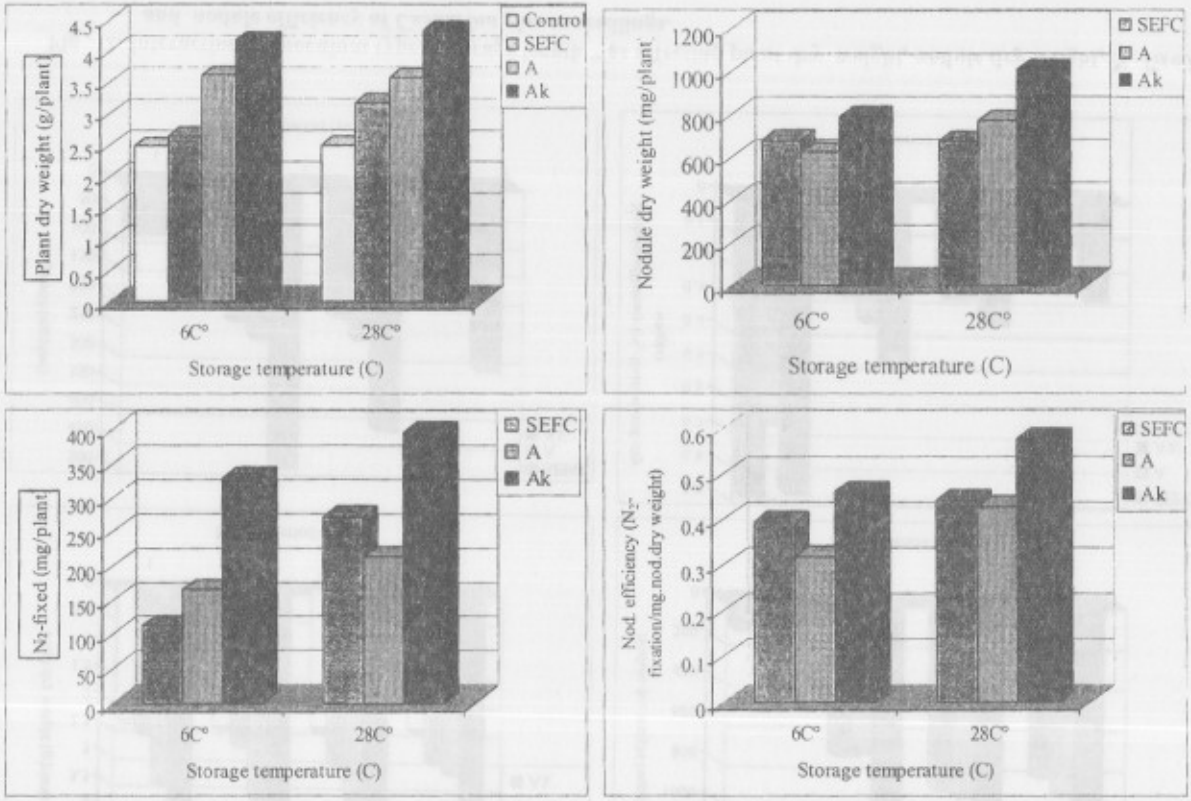

Fig . 3. Interactions " inoculum type x storage temperature" as affecting plant dry weight, nodule dry weight, N_2-fixed and nodule efficiency of *Casuarina glauca* seedlings.

Inoculant type/temperature/storage length interaction

The interaction of the 3 above-mentioned factors was highly significant with plant dry weight and N_2-fixation ($P<0.01$). While the maximum amounts of plant dry weight and nodule dry weights with AEIK inoculant were 4.75 and 1.45 g/plant after 3 and 6 months of storage at 28C° respectively, the maximum amounts of nodule efficiency and N_2-fixed with AEIK inoculant were 0.63 mg/plant and 1.25 (N_2-fixation/nod.dry weight) after the first and the third months of storage at 28C°, respectively.

Discussion

The technique for processing microbial inoculants used in agriculture is of great importance. The successful use of such inoculants depends not only on the survival of large numbers of microbial cells between production and application time, but also for long storage, proper transportation and ease of application. The production of inocula requires mass production of the target microorganisms to be introduced into the agrosystem. To date, only a few different methods of inoculation are used; the simplest and primitive inoculation method is the application of *Frankia* in liquid broth (Selim and Schwencke, 1994; Mansour *et al.*, 1996; Girgis *et al.*, 1990 and 2002). There are obvious logistical problems in applying this method in border scale (Periner *et al.*, 1985). The use of entrapped *Frankia* in alginate beads as an inoculant for field grown *Casuarina* was shown successful by Sougoufara *et al.* (1989). Instead of using an inoculant made of a *Frankia* culture merely entrapped in alginate beads, some studies proposed the possibility of improving this type of inoculant by incorporating some additive such as kaolinite with the alginate polymer. In this concern, some observations also showed the significance of clay in establishing effective *Frankia*-actinorhizal symbiosis in the field (Vogel and Dawson, 1985 and Righetti *et al.*, 1986; Girgis *et al.*, 1991).

A simple technique allowing production of high concentrations of *Frankia* spores is to cultivate *Frankia* for long incubation time (Diem and Dommergues, 1990), the use of certain specific carbon sources (Jiang and Zhu, 1985) or amino acids (Diem *et al.*, 1988) or to incubate *Frankia* cultures in a nitrogen-free liquid medium for 2 weeks (Diem *et al.*, 1988). Recently Girgis (2001), showed that high yield of spores were obtained at day 18 from stirring of nitrogen free media supplemented with 0.1% agar.

Assessment of cell viability in *Frankia* cultures is of particular importance in studies designed to improve cell culture methods (Prin *et al.*, (1990; Yang, 1995; Girgis, 2001 and 2002). The exponential growth and highest cell viability of *Frankia* growing in the first 10 days could be explained by the fact that the young culture was still composed of active hyphae. The oscillation of both growth and IRA of *Frankia* cultures after storage length 1, 3, 6, and 12 months either in room temperature (28°C) or in refrigerator (6°C) under static conditions, could be explained by the fact that, under these conditions heterogenous nature of the culture, young and old hyphae as well as sporangia and spores were found together (Fig. 1a).

Storage of AEIK and AEI *Frankia* inoculants in a low temperature (6°C) preserves the effectiveness up to 12 months and significantly increases plant dry weight, nodules number and dry weight. Burleigh *et al.* (1988) reported successful inoculation of *Alnus* and *Casuarina* seedlings using air-dried alginate beads stored at 4°C for periods of up to 2 years. However, Frankiae entrapped into alginate polymers can be stored for 2-3 years at 20-25°C without becoming infectiveless (Sougoufara *et al.*, 1989; Girgis, 1993).

The main effect of clay minerals on the soil microorganisms activity appeared to be related to modification of the physical and chemical characteristics of the microbial habitats which may stimulate the growth and metabolic activities of an individual microbial population (Stotzky and Burns, 1982). Stotzky (1985) also showed a possibility of direct surface interactions. Kaolinite has a pronounced influence due to its high cation exchange capacity (CEC), large surface area and swelling ability. Recently, Sayed *et al.*, (2002) recommended storage temperature for polyacrylamide gel (PAG)-immobilized *Frankia* in 7-28°C for up to 3 months. As shown in this investigation, both *Frankia* inocula entrapped into alginate or alginate plus kaolinite beads maintained high viability and infectivity up to 12 months when stored at 6°C or 28°C.

Obtained results in this work confirmed the previous data obtained by Girgis and Said (2002) that *Casuarina glauca* seedlings inoculated with *Frankia* entrapped into alginate-kaolinite carrier seemed to be good competitors to those inoculated with fresh with higher activity of the acetylene reduction activity (ARA).

It could be generally concluded that the response of *C. glauca* to inoculation by *Frankia* can be substantially improved by using inoculant technology based on polymer-entrapped *Frankia* as spore-enriched broth cultures. Since the production of such improved inoculants requires a certain level of expertise, pilot unites for inoculant production should be established in research centers.

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فاعلية ثلاث صور من لقاحات الفرانكيا تحت تأثير ظروف التخزين

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تم فى هـذا البحث دراسة تأثير طـول فترة التخزين ودرجة حرارة التخزين على نمو وحيوية وانتاج الجراثيم لمزارع الفرانكيا النامية فى المعمل فى فترات زمنية مختلفة . واوضحت النتائج ان أقصى كمية للبروتين الكلى للخلايا وأعلى مستوى لحيويتها فى المزارع النامية حتى اليوم العاشر من التلقيح. ولوحظ إنخفاض متدرج لكلا من النمو والحيوية بعد فترات تخزين شهر ، ٣، ٦، ١٢ شهر على درجة حـرارة ٢٨°م أو ٦°م. كما ظهر ان طول فترة التخزين ودرجة حرارة التخزين كان لهما تأثير ايجابى على معدل انتاج الجراثيم فى مزارع الفرانكيا الذى زاد جدا بعد مرور ١٠ ايام من التلقيح فى المزارع المحفوظة على درجة حرارة ٢٨°م. وكان اعلى معدل لكثافة الجراثيم بعد تخزين لمدة ٦ شهور على درجة حرارة ٢٨°م.

تلى ذلك اجراء تجربة أصص تحت ظروف الصوبة السلكية و ذلك لتقييم تاثير طول مدة و حرارة التخزين لثلاث انواع من لقاحـات الفرانـكيا على نمو و تعقيد و تثبيت الازوت الجوى لشتلات الكازوارينا جلوكا. وا للقاحات المختبرة هى: مزارع الفرانكيا الغنية بالجراثيم (SEC) ، الفرانكيا المحملة على الجينات الصوديوم (AEI) ، أو الجينات الصوديوم المضاف اليها كاوؤلين (AEIK) . و قد سجلت النتائج اختلافات معنوية بين أنواع اللقاحات المستخدمة فقد كان لقاح ال AEIK اكثر صور اللقاحات فاعلية سواء عند تخزينه على درجة حرارة ٢٨°م او ٦ °م. فقد ادى الى زيادة معنوية فى كلا من الوزن الجاف للنبات و العقد الجذرية و تثبيت الازوت .

و قد اوضح التفاعل بين انواع اللقاحات و فترة التخزين ارتفاع معنوية تأثير لقاح AEIK على الوزن الجاف للنبات ، والتعقيد وكذلك تثبيت الازوت فى جميع فترات التخزين المختبرة. و ثبت ان التفاعل بين درجة حـرارة التخزين و فترات التخزين لة تأثيرا معنويا على كلا من الوزن الجاف للنبات و تثبيت الازوت. اما بالنسبة لطول النبات و نشاط اختزال الاسيتيلين المتخصص (SARA) فكان تاثيرهم غير معنويا بالنسبة لكافة المعاملات.

و قد كان لتفاعلات العوامل الثلاثة المدروسة معا تأثيرا معنويا عاليا على نسبة الوزن الجاف للنبات و كذلك تثبيت الازوت الجوى. و على الرغم من انه تم الحصول على اعلى وزن جاف للنبات و تعقيد باستخدام لقاح AEIK بعد ثالث و سادس شهر من التخزين على درجة حرارة ٢٨°م فأنة تم الحصول على اعلى معدل لكفاءة العقد الجذرية و تثبيت الازوت الجوى بعد الشهر الاول و السادس من التخزين على نفس درجة الحرارة.