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LYOPHILIZATION AS A METHOD FOR PATHOGENS LONG TERM PRESERVATION

ABSTRACT: Lyophilization (freeze-drying) is one of the most suitable methods used for a long term peservation of pathogens. The aim of this paper was the application of lyophilization for storage of three significant plant pathogens: *Fusarium graminearum, Helmintosporium gramineum*, and *Pseudomonas syringae* pv. *gylicinea*, respectively. The plant material was collected continuously (during a four year period 2002—2006), depending on a plant development stage, from different localities in Vojvodina. Pathogens were isolated from diseased parts with characteristic symptoms, and placed on nutritive media specific for a certain pathogen, using standard phytopathological methods. Lyophilization was carried out in marked and coded ampoules by freezing and drying of pathogen suspension and nutritive medium. Revitalization of lyophilized isolates was done after four days. High percentage of revitalization was characteristic for all studied isolates, and it ranged from 85—92%, confirming that lyophilized pathogens would be capable of keeping viability for a long time in the collection. Besides above mentioned pathogens, there were 200 isolates in the collection, originating mostly from field and vegetable crops. Each isolate that was put into the Collection, was followed by all the necessary data such as: name of the pathogen, number of isolates, locality, host plant, year of isolation, name of the researcher and other relevant data.

KEY WORDS: lyophilization, long time preservation, plant pathogen collection, fungi, bacteria.

INTRODUCTION

The study of microorganisms often involves the use of living cultures. The cultures need to be kept for a long period, and included into the microbiological collection. Purpose of the Collection is to maintain the biological material in vital and stable state, with all its original traits preserved. Lyophilization (freeze-drying) is one of the most suitable techniques used for long-term pathogen preservation (The Preserevation and Maintenance of Living Fungi — Smith and Onions, 1994).

MATERIAL AND METHODS

The plant material was collected continuously, from different localities in Vojvodina, depending on a plant development stage. Pathogens were isolated from diseased parts with characteristic symptoms, and placed on nutritive media, specific for certain pathogens, using standard phytopathological methods. These isolates originated from different localities in Vojvodina (Futog, Begeč, Bačka Palanka, Rimski Šančevi, Čenej, Pančevo, Srbobran, Tovariševo, Njegoševo, Bačka Topola, Laćarak and Čalma, respectively) and were collected during the period 2002—2006.

Fusarium graminearum was isolated from diseased maize and soybean seed, and from diseased leaves and ear of barley. Standard PDA medium was used. The obtained fungi colonies, belonging to *Fusarium* genus, were subcultured on CLA medium for better sporulation (Fisher et al. 1982). Isolates were incubated at 25°C under artificial ultraviolet light ("black light") with a 12hr photoperiod.

Helminthosporium gramineum was isolated from diseased seed and leaf of barley and placed on standard PDA medium. Incubation was done at 25°C, with 12 hour light/dark cycle during seven days. The obtained isolates were transferred to PDA and WA media in order to obtain pure cultures.

On basis of fungus morphological characteristics of colonies and reproductive organs (conidia, conidiophores, and chlamidiospores), determination of species was done (Nelson et al., 1983; Burgess et al., 1994; Mathur and Kongsdal, 2003).

Pseudomonas syringae pv. *glycinea* was isolated from diseased soybean leaves with characteristic symptoms of bacterial spot, placed on meat extract (MPA) and nutritive agar medium, enriched with sucrose (NSA), using standard surface smearing method (Arsenijević, 1997; Schaad, 1980). Two days after development in thermostat at 26°C, individual colonies were transferred to slope medium with glycerol (Klement et al., 1990). Biochemical-physiological characteristics and pathogenic traits of the obtained isolates were determined by using tests and usual procedures for this kind of testing (Fahy and Persley, 1983; Arsenijević, 1988; Klement et al. 1990; Schaad, 2001).

Preparation of the isolates for lyophilization

F. graminearum and *H. gramineum* were lyophilized using colonies 7—10 days old, while bacterial colonies were 24—48 hours old.

Lyophilization was done according to Smith and Onions (1994). Prepared material was lyophilized in Modulyo 4K freeze dryer (Edwards, UK) under vacuum (Fig. 1). Lyophilization processed ampoules were constricted using flame, in order to prevent the contact of lyophilized material with air, which could cause damage (Rey, 1977) (Fig. 2).

Sealed ampoules were kept in a special compartment of lyophilization chamber.

Fig. 1 — Modulyo 4K freeze dryer (Edwards, UK)

Fig. 2 — Ampoule with lyophilized isolate

Viability of lyophilized isolates was checked four days after the lyophilization. Growing and morphological characteristics (shape, colour, and size) of fungal and bacterial colonies were observed on nutritive media during the revitalization process.

RESULTS

Ten isolates of *F. graminearum* fungus were obtained by isolation. Five isolates originated from soybean seed (FG-5, FG-8, 56/13, 4/21, 5/18), three from maize seed (FG-1, FG-2, FG-3), one from barley leaf (FG-6) and one from barley ear (FG-7).

Four isolates of *H. gramineum* originating from barley seed, were obtained (H-1, H-1 (F), HG-1 and HG-2), and one originating from barley leaf (HG-3).

Three to four days after the completion of lyophilization, 22 isolates of *P. syringae* pv. *glycinea* (B2/4, B2/5, B2/6, B2/7, B2/8, B11/1, B13/1, B13/2, B13/4, S5/1, S5/2, S5/3, R9/1, R9/3, R10/1, R10/4, R10/5, R10/6, R12/2, P15/1, P15/2 i P15/3) were obtained.

Besides the above mentioned pathogens, the Collection contains 200 isolates originating mainly from field and vegetable crops. Each isolate contained in the Collection was put into the data base with all the relevant data, such as name of the pathogen, number of isolates, locality, host plant, year of isolation, name of the researcher and other relevant data (Tab. 1).

Tab. 1 — Form of table containing data on isolates and localities

Catalog number of isolate	
Name of pathogen	
Locality	
Host and part of plant used for isolation	
Year of introduction of isolate into the Collection	
Other data: name of institution, name of researher, original mark of isolate, origine of isolate, year of isolation and other relevant data	

Percentage of revitalized isolates four days after lyophilization was 85% for *F. graminearum* and *H. gramineum* fungi, and 92% for *P. syringae* pv. *glycinea* bacterium. These results point out to a high degree of isolate viability after lyophilization, confirming that the studied pathogens are capable of long term preservation by application of this method.

Morphological and growing characteristics of the colonies of revitalized fungal and bacterial isolates, observed on nutritive media, were identical with the original. On PDA, medium isolates of *F. graminearum* formed abundant, thick, and pinkish white mycelia, with grayish margines. The isolates formed burgundy pigment in the agar. On CLA medium, the isolates formed pink aerial mycelium, and dark red pigment in the agar. Macroconidia were formed on branched monophialides with 3—5, rarely 6 clearly visible septa. Apical macroconidial cell was tapered, while basal cell was foot-shaped. The isolates formed neither microconidia nor chlamidospores. (Fig. 3).

H. gramineum formed grayish to olivegreen black colonies with characteristic lobed margins, after revitalization on PDA medium. Conidiophores were branched and septate, formed individually or in groups. Cylindrical dark yellow conidia with 1—7 septa were formed laterally or terminally on conidiophores (Fig. 3).

Bacteria were revitalized on nutritive medium enriched with sucrose (NSA). Characteristic slimy, large, shiny, white and distinctively convex bacterial co-

Fig. 3 — Revitalized isolates of *H. gramineum* and *F. graminearum* on PDA medium

lonies of *P. s.* pv. *glycinea* were formed three to four days after revitalization (Fig. 4).

Fig. 4 — Revitalized isolate of *P. s.* pv. *glycinea* bacterium on NSA medium

DISCUSSION

Plant pathogens including *F. graminearum*, *H. gramineum* and *P. s. pv. glycinea* can cause significant damage to the plant production, and for that reason they should be precisely determined and preserved. Plant pathogens, which are the part of agro-ecosystem, are influenced by great number of factors, causing the change of their characteristics among which pathogenicity and virulence are the most significant ones. This fact revealed the need for a systematic collection and a long-term preservation of pathogens. Due to the changes caused by different conditions of environment, it is very important, especially from the aspect of breeding for resistance, to have a great number of isolates of a certain pathogen originating from different localities and different years. Lyophilization method enabled the biological material to preserve its original traits for long periods of time (Klement, 1990; Agrios, 1997).

Results obtained in our studies confirmed that the method of lyophilization is a very suitable way for plant pathogen preservation.

Microorganisms can survive conditions of lyophilization process, and preserve viability and original traits. Success of lyophilization and vitality of lyophilized isolates can vary between the isolates of the same species (Smith and Onion, 1994). Tan et al. (1991) mentioned that the optimal results can be obtained in case of lyophilization of young colonies, and that this method is proposed for preservation of fungi belonging to *Ascomycetes* class, to which *H. gramineum* and *F. graminearum* also belong.

The length of time allowed for rehidratation is one of the major factors in the process of revitalization, and can be very different for individual isolates (Haskins, 1957, Butterfield et al. 1974).

All studied isolates showed high percentage of revitalization and survival. Morphological and growing characteristics of lyophilized *F. graminearum* and *H. gramineum* fungi, and *P. s.* pv. *glycinea* bacterium, were identical to the original isolates after revitalisation.

The need for constant observation, study and preservation of plant pathogens, with the aim of improvement of cultivated plant production, first of all by developing less sensitive i.e. resistant varieties, genotypes, and hybrids comes from all the above mentioned.

REFERENCES

Agrios, G. N. (1997): *Plant Pathology*, Fourth Edition. Academic Press, San Diego, California, USA.

Arsenijević, M. (1988): *Bakterioze biljaka*, Naučna knjiga. Beograd.

Arsenijević, M. (1997): *Bakterioze biljaka*, S print. Novi Sad.

Burgess, L., Summerell, B., Bullock, S., Gott, K., Backhouse, D., (1994): *Laboratory Manual for Fusarium Research*, Third edition, Sydney.

Butterfield, W., Jong, S. C., Aleksander, M. J. (1974): *Preservation of living fungi pathogenic for man and animals*, Canadian Journal of Microbiology 20, 1665—1673.

Fahy, P. C., Persley, G. J. (1983): *Plant Bacterial Diseases, A Diagnostic Guide*, Academic Press, Australia.

Fisher, N., Burgess, L., Toussoun, T., Nelson, P., (1982): *Carnation leaves as a substrate for preserving cultures of Fusarium species*, Phytopathology 72: 151—153.

Haskins, R. H. (1957): *Factors affecting survival of lyophilized fungal spores and cells*, Canadian Journal of Microbiology 3, 477—485.

Klement, Z., Rudolph, K., Sands, D. C. (1990): *Methods in Phytobacteriology*, Akademiai Kiado. Budapest.

Machado, J., Langerak, C., Jaccoud-Filho, D. (2002): *Seed-Borne Fungi A contribution to Routine seed health Analysis*, ISTA, Zürich.

Mathur, S. B., Kongsdal, O. (2003): *Common Laboratory Seed Health Testing Methods for Detecting Fungi*, ISTA, Zürich.

Nelson, P., Toussoun, T., Marasas, W. (1983): *Fusarium species An Illustrated Manual for Identification*, The Pennsylvania State University Press.

Rey, L. R. (1977): *Glimpses into the fundamental aspects of freeze drying*. In: *Development in Biological Standardization* (edited by V. J. Cabasso & R. H. Regamey) Basel: S. Krager.

Schaad, N. (1980): *Laboratory guide for identification of plant pathogenic bacteria*, APS Press, St. Paul, Minnesota, USA.

Schaad, N., Jones, J. B., Chun, W. (2001): *Laboratory guide for identification of plant pathogenic bacteria*, APS Press, St. Paul, Minnesota, USA.

Smith, D., Onions, A. H. S. (1994): *The Preservation and Maintance of Living Fungi*, Second Edition, CAB International, UK.

Tan, C. S., Ingen, C. W. van Stalpers, J. A. (1991): *Genetics and breeding of Agaricus*, Proceedings of the First International Seminar on Mushrooms Science, Mushroom Experimental Station, Horst, Nederlands, 14—17 May, 25—30.

ЛИОФИЛИЗАЦИЈА КАО МЕТОД ДУГОРОЧНОГ ЧУВАЊА ПАТОГЕНА

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Резиме

Лиофилизација (freeze-drying) је једна од најпогоднијих метода која се користи за дугорочно чување патогена. Циљ рада је примена метода лиофилизације у чувању три значајна биљна патогена: *Fusarium graminearum, Helminthosporium gramineum* и *Pseudomonas syringae* pv. *gylicinea*. Прикупљање узорака биљног материјала вршено је у континуитету (током четири године, 2002—2006) са различитих локалитета на подручју Војводине, у зависности од развојне фазе биљака. Изолација патогена вршена је из оболелих биљних делова са карактеристичним симптомима на хранљиве подлоге специфичне за одређеног патогена коришћењем стандардних фитопатолошких метода. Поступак лиофилизације вршен је у обележеним и шифрираним ампулама смрзавањем и сушењем суспензије патогена и хранљивог медијума. Ревитализација лиофилизованих изолата извршена је четири дана након лиофилизације. За све проучене изолате карактеристичан је висок проценат ревитализације и износи 85—92%, што потврђује да ће лиофилизовани патогени дугорочно задржати виталност у колекцији.

У оквиру колекције поред наведених патогена постоји 200 изолата који потичу углавном са ратарских и повртарских биљних врста. Сваки изолат који се налази у колекцији унет је у базу података са свим потребним подацима, као што су: назив патогена, број изолата, локалитет, биљка домаћин, година изолације, име истраживача и други релевантни подаци.