International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 8 | Issue 2 | APR-JUN | 2018 | 634-638
Research Article | Biological Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

RAPID STAINING FOR ISOLATION OF FUNGAL ENDOPHYTES FROM LEAVES OF FOUR CHILI VARIETIES OF ASSAM

I Barman[1*], J Gogoi[1] and AM Dutta[2]
[1]Department of Biochemistry, Assam down town University, Panikhaiti, Guwahati, Assam
[2]Department of Chemistry, Assam down town University, Panikhaiti, Guwahati, Assam

*Corresponding Author Email: rcheindrani@gmail.com

ABSTRACT

Fungal endophytes occupy around 41% of the endophytic community and are an unexplored trove of endosymbionts that lives within a plant for at least part of its life cycle without harming the host plant. Due to their chemical diversity, they have profound roles in promotion of plant growth, high yielding rate and biological control agent. But the irony lies in their rapid and effective isolation techniques since they are internal to plant tissues. Therefore, the present study was designed to detect the presence of endophytes in the different varieties of chili leaves (Capsicum annum L., Capsicum chinense Jacq., Capsicum assamicum J. Pur – L. Singh and Capsicum frutescens L.). Fresh and uninfected abaxial peel of the leaves were stained and observed under light microscope by the two different staining procedures. The same tissue was cultured on PDA plates after surface sterilization. Spherical shaped spores were distinctly stained red by using rose bengal stain and produced slimy layer endophytes on PDA plates. Irregular rod-shaped spores were distinctly stained with both the dyes, but lactophenol-trypan blue dye was found to be more suitable for staining these irregular rod-shaped spores and produced mat type endophytes on PDA plates. Therefore, by using these two staining techniques, one can validate probable fungal endophytes which was found on culturing the same tissue on the culture plates.

KEY WORDS

Endophytes, Isolation, Lactophenol – Trypan Blue Stain,Rose Bengal Stain

INTRODUCTION:

Chili plants have been already reported for the presence of endophytic fungi within them [1]. Endophytic fungi are the organisms living internally in tissues of the plants without causing any apparent symptoms [2] comprising both commensal species and mutualistic symbionts [3]. These are polyphyletic; mostly belonging to ascomycetes and to anamorphic fungi [4, 5]. Reports have revealed these diversified fungi offering wide roles in plant protection, acting against herbivores, insects and pathogens of the host, increase plant resistance to pathogens and biotic and abiotic stresses [6,7]. These are also rich sources for bioactive antimicrobial compounds such as alkaloids, peptides, steroids and phenol, which have a wide range of applications in the medical field [8]. However, methodological limitations have limited the study of these endophytic groups and the major challenge being the isolation of these endophytes keeping in mind the fact that these are found in internal plant structures.

Endophytic study is typically a method-dependent process. To a wide extent, their detection and isolation basically involves microscopic examination based on morphological characters, cultivation techniques using different media compositions, and finally molecular taxonomic tools [9]. But these methods carry along their respective advantages and disadvantages where the restriction goes to the choice of technique and biased results in favour of certain taxa [10].

Surface sterilization followed by plate culture method alone will not serve a reliable isolation of fungal

endophytes. The greater irony being time consuming, lack of sensitivity and specificity, slow, laborious and difficulty in interpretation and most importantly numerous endophytes may remain unisolated [11]. So, a prior microscopic visualization using differential staining of the various plant parts followed by its plate culture could prove effective to minimise its limitations. Staining techniques and microscopic examination based on differentiating hyphae are key towards fundamental mycology, plant pathology and related horizons [12]. Kuldau and Yates (2000) revealed the precise internal locations of endophytic fungi using light and electron microscopy. These staining techniques may vary from simple single stain to complex multi-stain procedures [13]. Plant parts like epidermal peel from leaf sheath, pith scrapings and seeds have been examined using various stains [14]. Lacto Phenol method can stain the fungi blue aiding in easier visualization and examination [15] whereas Cotton blue stains the fungal protoplasm without staining the cell walls thereby exposing the septa of spores as well as mycelium clearly visible [16]. For the staining of mycelium, lactophenol trypan blue [17] and aniline blue [18] are employed where trypan blue stains hyphae better. A general histological stain, gentian violet is a simple and clear cut stain which does not provide differential staining [16].For detection of endophytic fungi in plant tissues (including seeds), rose Bengal stain is advantageous in terms of better visualization and faster (30-60 sec) as well as safer than trypan blue (3-5 min) [14].Apart from these, toluidine blue O, safranin& fast green staining, pianese III B stain, KOH aniline blue are used to localise endophytic fungi within the leaves, chlorazole Black E stains within the roots and rhodamine B / methyl green method within the plant woods[19].

Therefore, the objective of this study was to compare the rapid and effective staining using lactophenol trypan blue and rose bengal stain in leaves of four different chili varieties (*Capsicum annum* L., *Capsicum chinense* Jacq., *Capsicum assamicum* J. Pur – L. Singh and Capsicum *frutescens* L.) and to correlate the results of endophytic fungal structures observed in the staining method with the results of endophytic fungal structures grown on the media plate.

MATERIALS AND METHODS:

Collection of sample:

Leaf sheath of four different chili varieties of Assam (*Capsicum annum* L., *Capsicum chinense* Jacq., *Capsicum assamicum* J. Pur – L. Singh and *Capsicum frutescens* L.) were collected from Kundilnagar area of Narengi, Assam. Fresh and uninfected leaf samples were used in this study.

Staining Techniques:

Rose Bengal Stain:

A standard solution of Rose Bengal was prepared: 0.5% rose Bengal dissolved in 5% aqueous ethyl alcohol. The shelf life of the standard solution is 5-6 months. Firstly, the leaf was rinsed with distilled water and dried. Leaf sheaths of the selected varieties were opened and a thin layer on the inside was peeled abaxially, three to four peeled sections were placed on a glass slide, and one to two drops of standard rose Bengal solution was applied to the samples. Staining time was usually 30-60 sec but could be as short as 15 seconds in well peeled samples. Samples were covered with cover slip and excess stain was then drawn off [14]. All preparations/slides were examined under bright field microscope.

Lactophenol Trypan Blue Stain:

The lactophenol contains the following reagents in the ratio of 1:1:1:5 - lactic acid, glycerol, liquefied phenol and water. Epidermal tissue was peeled from the leaf sheath and placed on a glass slide. Three to four drops of lactophenol -0.1% trypan blue solution was added and kept for 3-5 minutes. The slide was gently heated over flame and 1-2 drops of water was added. The excess stain was removed, and a cover glass was placed over the stained samples [14]. All preparations/slides were examined under bright field microscope.

Triplicate samples were tested for both trypan blue and rose Bengal stain.

Isolation using Plate culture of the same stained leaf tissue:

To validate the probable fungal endophytes, the same stained leaves were surface sterilized with distilled water, 70% ethanol, 3% NaClO using standard procedure. The dried parts were carefully placed on potato dextrose agar (PDA) petri plates and incubated at 28-degree celsius for 72 hours [20].

Triplicate sets of plate culture were performed.

RESULTS AND DISCUSSION:

Staining techniques:

The endophytic fungal development in different chili varieties by the penetrating hyphae was observed using bright field microscope. Hyphae were the principal structures observed. Lactophenol trypan blue stained blue and rose Bengal cotton blue stained red in contrast with the light green of the plant leaves. Our results showed that both lactophenol trypan blue staining and rose Bengal staining were quick and effective method for staining endophytic fungi in plant tissues. Two types of fungal spores were identified by using both the stains namely, Lactophenol –Trypan Blue and Rose Bengal Stain. Spherical shaped spores and irregular rod-shaped spores were principally seen in the various chili varieties.

Out of these four chili varieties, three varieties namely *C.assamicum* and *C.annuum* and *C.frutescens* showed the presence of both spherical shaped spores and irregular rod shaped spores. *C.chinense* exhibited the presence of only one type of spore i.e spherical shaped spores.

Among the two tested stains, spherical shaped spores were distinctly stained red by using rose Bengal stain whereas, by using lactophenol-trypan blue stain, spherical spores were not stained uniformly. Irregular rod-shaped spores were distinctly stained with both the dyes, but lactophenol-trypan blue dye was found to be more suitable for staining these irregular rod-shaped spores.

Isolation by plate culture method:

The plate culture methods revealed correlated results with the microscopic examination for fungal endophytes. In the culture dependent method, *C.assamicum* showed the presence of both white mat fungal growth and slimy layer, which clearly correlates to the irregular rod shaped and spherical shaped spores respectively seen in the microscopic staining method(figure 1a-c). *C.annuum* also showed interesting results that revealed the presence of both white mat and green mat type as already seen as irregular rod shaped microscopically (figure 3a-c). In addition, *C. frutescens* (small green var. and green black var.) also exhibited the presence of white mat and slimy layer respectively as observed in staining method (figure 2a-c, 3a-c). In contrast to these three varieties, *C.chinense* which revealed only the presence of spherical shaped spores showed only slimy layer, which totally correlated the two methods, staining method and culture dependent method (Figure 1).

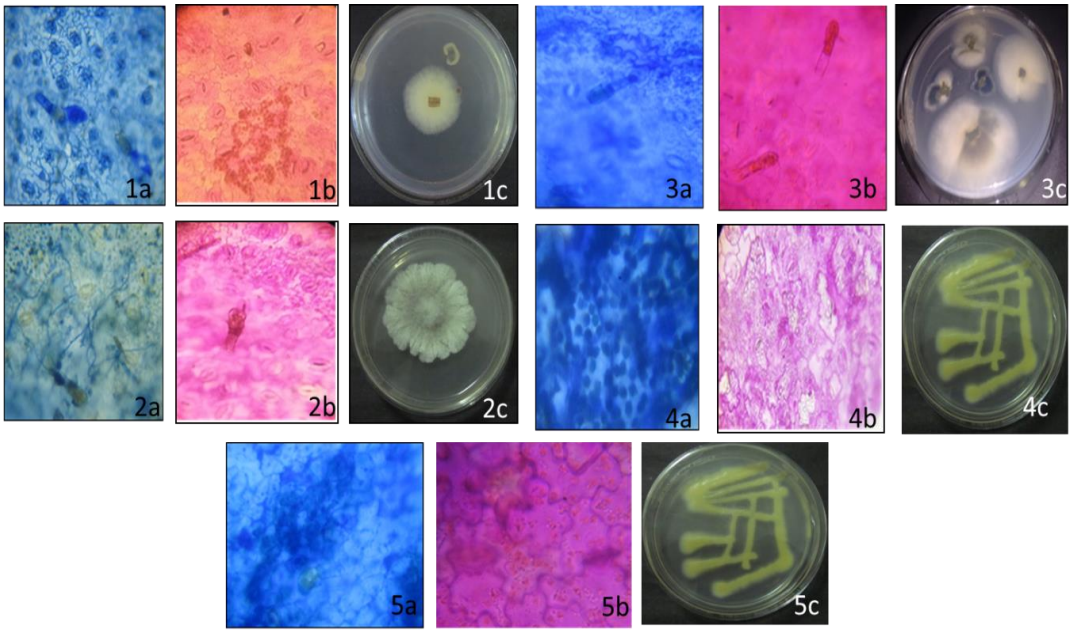

Figure 1 Staining of abaxial surface peel of leaves of four different chili varieties. Lactophenol –Trypan Blue and Rose Bengal Stains were used to stain spores of endophytes. Rose bengal stained spores red and Lactophenol stained dark blue. (1a – 1c) is *Capsicum assamicum* J. Pur – L. Singh L. , (2a – 2c) is *Capsicum frutescens* L.(small green var.) , (3a – 3c) is *Capsicum annum* L, (4a – 4c) is *Capsicum frutescens* L (green black var.) and (5a – 5c) is *Capsicum chinense* Jacq.

CONCLUSION:

For detection of endophytic fungi from different plants, differential staining procedures are reported earlier [21-25]. But there is a dearth of knowledge related to microscopical studies of endophytes especially fungal endophytic assemblage within the leaves of various chili varieties of Assam. However microscopic data needs further well supportive evidences through molecular assessment. PCR techniques could clearly figure out the microscopic results to evaluate the presence as well as colonisation of the endophytes. Culture-independent molecular methods have been reported useful to evaluate plant endophytic communities [26]. Comparing the traditional culture-based techniques, the DNA-based assessments might serve advantageous in endophytic identification which are difficult to culture in vitro [27]. Hence, these dual combinatorial approaches could probably be employed in various investigations of this kind so as to properly ensure the actual endophytic fungal localisation in different parts of chili varieties.

ACKNOWLEDGEMENT:

The authors acknowledge Assam down town University, Panikhaiti for the financial and laboratory facilities in carrying out this study.

CONFLICT OF INTEREST: None

REFERENCES:

1. Hernawati H, Wiyono S, Santoso S. Leaf endophytic fungi of chili (*Capsicum annuum*) and their role in the protection against *Aphis gossypii* (Homoptera: Aphididae), Biodiversitas, 12(4):187-191, (2011).
2. Stone JK, Bacon CW, White JF. An overview of endophytic microbes: endophytism defined. In: Bacon CW, White JF (eds.), Microbial endophytes, Marcel Dekker Inc., NewYork,3-29, (2000).
3. Strobel G, Daisy B. Bioprospecting for microbial endophytes and their natural products. Microbiology and Molecular Biology, 67(4): 491-502, (2003).
4. Arnold AE, Lewis LC. Ecology and evolution of fungal endophytes, and their roles against insects. In: Vega FE, Blackwell M (eds.), Insect-Fungal Associations: Ecology and Evolution, Oxford University Press, New York, 74-96, (2005).
5. Tan R X and Zou W X. "Endophytes: a rich source of functional metabolites". Natural Product Report,18: 448-459, (2001).
6. Pimentel IC, Glienke-Blanco C, Gabardo J, Stuart RM and Azevedo JL. Identification and Colonization of Endophytic Fungi from Soybean (Glycine max (L.) Merril) under Different Environmental Conditions. Brazilian Archives of Biology and Technology, 49(5):705-711, (2006).
7. Procopio REL, Araujo WL, Maccheroni WJ and Azevedo JL. Characterization of an endophytic bacterial community associated with Eucalyptus spp. Genetics and Molecular Research, 81408-1422, (2009).
8. Anitha D, Vijaya T, Pragathi D, Reddy NV, Mouli KC, Venkateswarulu N andBhargav DS. Isolation and characterization of endophytic fungi from endemic medicinal plants of tirumala hills. International Journal of LifeSciences Biotechnology and Pharma Research, 2(3), (2003).
9. Hyde KD, Soytong K.The fungal endophyte dilemma. Fungal Diversity, 33:163–173, (2008).
10. Schulz B and Boyle C.The endophytic continuum. Mycological Research, 109: 661-686, (2005).
11. Stroble G, Daisy B, Catillo U. Novel natural products from rainforest endophytes. In: Natural products: Biodiversity, chemical diversity and drug discovery, Part V (eds. L Zhang, AL Demain). Humana press Inc. Totoea, NJ, 329–351, (2005).
12. Aly AH, Debbab A, Kjer J, Proksch P. Fungal endophytes from higher plants: a profilic source of phytochemicals and other bioactive natural products. Fungal Diversity, 41:1–16, (2010).
13. Zhang HW, Song YC, Tan RX. Biology and chemistry of endophytes. Natural Product Report, 23(5): 753–771, (2006).
14. Xu L, Zhou L, Zhao J, Jiang W. Recent studies on the antimicrobial compounds produced by plant endophytic fungi. Natural Product Research and Development,20: 731–740, (2008).
15. Rozin P and Schiller D. The nature and acquisition of a preference for chili pepper by humans. Motivation and Emotion, 4:77−101, (1980).
16. Perry L, Dickau R, Zarrillon S, Holst I, Pearsal DM, Piperno DR, Berman MJ, Cooke RG, Rademaker K,Ranere AJ, Raymond JS, Sandweiss DH, Scaramelli F, Tarble K and Zeidler JA. Starch fossils and the domestication and dispersal of chili peppers (Capsicum spp.L.) in the Americas. Science (Reports), 315:786−788, (2007).
17. Govindappa M, Murthy SN, Shwetha S, Akshatha RV, Channabasavaand Ravishankar RV. Determination ofcapsaicinoids from endophytic fungi (*Aspergillusniger*, *Alternaria sp.*and*Penicillium sp*.) of*Capsicum annuum* using LC-MS. Plant Sciences Feed,5(2, (2015).
18. 18.Bosland PW. andVotava EJ. Peppers: Vegetable and spice capsicums. CABI Publishing, Wallingford, UK, 23-29, (1999).

19. Sampson K. The systemic infection of grasses by*Epichloetyphina*(Pers.) Tul, Transactions of the British Mycological Society, 18(1): 30-47, (1933).
20. Souza A, Cruz JC,Sousa NR, Procópio ARL, Silva GF. Endophytic bacteria from banana cultivars and their antifungal activity, Genetics and Molecular Research, 13(4): 8661-8670, (2014).
21. Petrini O, Fischer PJ.Occurrence of fungal endophytes in twigs of *Salix fragilis* and *Quercusrobur*. Mycological Research, 94:1077–1080, (1990).
22. Kowalski T, Kehr RD. Endophytic fungal colonization of branch bases in several forest tree species. Sydowia, 44:137–168, (1992).
23. Baum S, SieberTN, Schwarze FWMR, Fink S. Latent infections of Fomesfomentariusin the xylem of European beech (*Fagussylvatica*). Mycological Progress, 2:141–148, (2003).
24. Gomez-Vidal S, Lopez-Llorca LV, Jansson HB, Salinas J. Endophytic colonization of date palm (*Phoenix dactylifera* L.) leaves by entomopathogenic fungi. Micron, 37:624–632, (2006).
25. Garcia A, Rhoden SA, Rubin Filho CJ, Nakamura CV, Pamphile J. Diversity of foliar endophytic fungi from the medicinal plant *Sapindussaponaria*L. and their localization by scanning electron microscopy. Biological Research, 45:139–148, (2012).
26. Guo LD, Hyde KD, Liew ECY. Detection and taxonomic placement of endophytic fungi within frond tissues of *Livistonachinensis*based on Rdnasequences.Molecular Phylogenetics and Evolution, 20:1–13, (2001).
27. Gao -X-X, Zhou H, Xu D-Y, Yu C-H, Chen Y-Q, Qu L-H. High diversity of endophytic fungi from the pharmaceutical plant, *Heterosmilax japonica*Kunth revealed by cultivation-independent approach. FEMS Microbiology Letters, 249:255–266, (2005).

***Corresponding Author:**
I Barman*
Email: **rcheindrani@gmail.com**