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Diagnosis of Potato Leafroll Disease by Fluorescence Microscopic Detection of Callose Stained with Resorcin Blue

Cheol Ho Lee* and Yong Joon La[1]
Department of Biological Engineering, College of Natural Sciences and Technology, Seokyeong University, Seoul 136-704, Korea
[1]Department of Agricultural Biology, College of Agriculture and Life Sciences, Seoul National University, Suwon 441-744, Korea

Resorcin Blue 염색 기법에 의한 감자 잎말림병의 형광 현미경적 진단

이철호* · 나용준[1]
서경대학교 이공대학 생물공학과, [1]서울대학교 농업생명과학대학 농생물학과

ABSTRACT : Deep blue fluorescence of resorcin blue-stained callose was observed only in the potato leafroll virus (PLRV)-infected potato plants, but not in other potato viruses investigated. The plant sections stained with aniline blue showed non-specific fluorescence regardless of PLRV infection, which means that aniline blue is not suitable for the staining of callose induced by PLRV infection. The fluorescence of resorcin blue-stained callose was more easily detectable than autofluorescence by a direct fluorescence detection method because of its deep blue color. The lateral branch of lower leaves was turned out to be the best material for fluorescence observation of all plant parts tested. In comparison of diagnostic efficacy of this technique to enzyme-linked immunosorbent assay (ELISA), PLRV-infected potato plants showed corresponding increment of the fluorescence of resorcin blue-stained callose as absorption values in ELISA increased. In the future, the criteria measuring the fluorescence objectively are thought to be determined for the practical application to the diagnosis of potato leafroll disease.

***Key words* : diagnosis, potato leafroll disease, deep blue fluorescence, resorcin blue, ELISA.**

Potato leafroll is one of the most important viral diseases of potato in many countries and yields of infected plants may be reduced by as much as 50% (5, 15). Control of the disease has depended primarily on the use of virus-free seed potato and elimination of aphid vectors (5, 15, 18). Despite the recent development of accurate and highly sensitive diagnostic methods such as enzyme-linked immunosorbent assay (ELISA) (2, 10, 23, 25), dot-ELISA (20) and immunosorbent electron microscopy (ISEM) (14), lack of more rapid and convenient one greatly hampers such seed potato certification schemes as well as to some extent breeding programs for the production of virus-free or resistant cultivars (16, 21).

Since the first study of de Bokx (4) on callose test, many researchers reported that the excessive accumulation of callose substance could be used as an indicator of PLRV infection (8, 11, 12, 22, 24). However, this technique is not fully reliable and is less sensitive than the above mentioned diagnostic techniques. In this point of view, Namba *et al.* (17) developed the direct fluorescence detection (DFD) method as the advanced technique of callose test and stated that the DFD method is much more sensitive than the light microscopic detection of callose. However, we found that the DFD method needs more technical improvement for the practical application such as technique for the discrimination between callose-specific fluorescence and non-specific one.

The present study was carried out to determine the optimum conditions for diagnosis of potato leafroll disease by the fluorescence microscopic detection of

*Corresponding author.

callose stained with resorcin blue. Its diagnostic efficacy was also compared to that of enzyme-linked immunosorbent assay.

MATERIALS AND METHODS

Potato plants, potato viruses and ELISA kits. Plant materials were obtained from the Alpine Experiment Station and the Horticultural Experiment Station and stored at −20°C in polyethylene bags or grown in the greenhouse for further work. PLRV-infected potato plants were kindly provided by Dr. Young Il Hahm in the Alpine Experiment Station, Daekwallyeong, Korea. Characteristics and varieties of the plants used in this study were summarized in Table 1.

Pure cultures of potato virus X, Y, S and M, and ELISA kits were obtained from the Alpine Experiment Station and the International Potato Center, Lima, Peru, respectively.

Preparation of plant materials for fluorescence microscopy. The pure cultures of potato virus X, Y, S and M were mechanically inoculated onto the upper leaves of the meristem-cultured healthy potato plants. Fourteen days after inoculation, upper, middle and lower leaves were harvested, of which three plant parts, midrib, petiole and lateral branch, were excised and fixed in each of the following : 5% glutaraldehyde (Sigma Chemical Co., St. Louis, Missouri) for 1 hr, formaldehyde acetic acid (FAA) (5 ml of formalin and glacial acetic acid dissolved in 90 ml of 80% ethanol) for 30 min or boiling tap water for 8 min. These were cross-sectioned 0.5~0.8 mm in thickness with a razor blade and applied to staining procedures as follows: Place the sections in 1% aqueous solution of resorcin blue (Sigma) for 15 min or 0.1% aniline blue (Sigma) for 5 min, wash in 3 changes of distilled water and blot the sections on the filter paper. The samples for the DFD method were prepared by the method of Namba *et al.* (17). The PLRV-infected and meristem-cultured healthy potato plants were also treated in the same manner as above.

Table 1. Plant status and varieties of potato plants used

Plant status	No. of potato plants used				
	Summi	Namjak	Daeji	Daewon	Total
Healthy-looking[a]	8	10	5	2	25
PLRV-diseased[a]	12	12	20	5	49
PLRV-infected[b]	4	4	–	–	8
Meristem-cultured healthy	4	4	12	–	20

[a] Indexed through visual inspection.
[b] Meristem-cultured healthy potato plants inoculated with PLRV.

Fluorescence microscopy. Stained sections were observed under a Vicker M17 fluorescence microscope with reflected light and filter combination of exciter filter MUG2 and barrier filter GG400+GG420. Kodak films (ASA 400) were used for photography.

Enzyme-linked immunosorbent assay. Potato leaves were macerated with mortar and pestle in 0.1 M phosphate-buffered saline (pH 7.4) containing 0.05% Tween-20 and polyvinyl pyrrolidone (40 kD, Sigma). After centrifugation at 3,000 rpm for 5 min, proper volume of resultants was applied to ELISA procedures. ELISA was carried out according to the recommended protocols of the International Potato Center.

RESULTS

The fluorescence specific to callose stained with resorcin blue was observed only in the PLRV-infected potato plants. The PLRV-infected potato plants, regardless of detection methods, showed a strong positive fluorescence reaction (Table 2). However, potato plants inoculated with potato virus X, Y, S or M showed no fluorescence reaction by resorcin blue staining or direct method. As shown in Table 2, both virus-infected and healthy potato plants showed positive fluorescence reaction when stained with aniline blue, although the intensity of fluorescence in the PLRV-infected potato plants was stronger than that in the other virus-infected and healthy potato plants.

Optimum conditions for observation of specific fluorescence of callose were determined. To define the best conditions for observation of specific fluorescence of callose caused by PLRV, the effects of plant parts, fixation and staining methods on the fluorescence reaction were examined. When the fluorescence reaction in different parts of the PLRV-infected potato plants was compared, the lateral branch of lower leaves gave the best results, as shown in Table 3.

All the PLRV-infected potato plants showed the strong fluorescence reaction regardless of the fixation methods tested (Table 4). However, the fluorescence reaction of PLRV-infected potato plants was stronger in the tissues fixed with FAA or boiling tap water than in those with 5% glutaraldehyde or no fixation. The

Table 2. Fluorescence reaction in phloem tissues of potato plants infected with different potato viruses

Potato virus	Degreee of fluorescence[a]		
	Auto-fluorescence[b]	RBSF[c]	ABSF[d]
Potato virus X	−	−	+
Potato virus Y	−	−	+
Potato virus S	−	−	+
Potato virus M	−	−	+
Potato leafroll virus	++	++	+++
Control[e]	−	−	+

[a] Observed 14 days after inoculation, without fixation; − : no fluorescence, + : weak fluorescence, ++ : moderate fluorescence, +++ : strong fluorescence.
[b] Observed by the method of Namba *et al.*
[c] Observed after resorcin blue staining.
[d] Observed after aniline blue staining.
[e] Meristem-cultured healthy potato plants.

Table 3. Fluorescence reaction in phloem tissues of different plant parts of the PLRV-infected potato plant

Plant parts		Degree of fluorescence[a]	
		Auto-fluorescence[b]	RBSF[c]
Upper leaves	Midrib	+	+
	Petiole	+	+
	Lateral branch	+	++
Middle leaves	Midrib	+	+
	Petiole	+	+
	Lateral branch	+	++
Lower leaves	Midrib	+	++
	Petiole	+	+
	Lateral branch	++	+++

[a] Fresh tissues were observed without fixation; + : weak fluorescence, ++ : moderate fluorescence, +++ : strong fluorescence.
[b] Observed by the method of Namba *et al.*
[c] Observed after resorcin blue staining.

Table 4. Effects of fixation methods on the fluorescence in the phloem tissues of PLRV-infected or meristem-cultured healthy potato plants

Plant status and variety	Degree of fluorescence[a]			
	NF[b]	GA[c]	FAA[d]	BTW[e]
PLRV-infected				
Summi	++	++	+++	+++
Namjak	++	++	+++	+++
Meristem-cultured healthy				
Summi	−	−	+	+
Namjak	−	−	+	+
Daeji	−	−	+	+

[a] Lateral branch of lower leaves was observed after resorcin blue staining; − : no fluorescence, + : weak fluorescence, ++ : moderate fluorescence, +++ : strong fluorescence.
[b] Non-fixed.
[c] Fixed in 5% glutaraldehyde for 1 hr.
[d] Fixed in formaldehyde acetic acid for 30 min.
[e] Fixed in boiling tap water for 8 min.

Table 5. Comparison of several diagnostic techniques dependent on callose specific fluorescence

Plant status	No. of plant tested	No. of plants showing fluorescence[a]		
		DFD[b]	RBSF[c]	ABSF[d]
Healthy-looking[e]	25	1	7	23
PLRV-diseased[e]	49	29	36	48
PLRV-infected[f]	8	8	8	8
Meristem-cultured healthy	20	0	0	14

[a] The plants with moderate fluorescence were counted positively.
[b] Observed by the method of Namba *et al.*
[c] Observed after resorcin blue staining.
[d] Observed after aniline blue staining.
[e] Indexed through visual inspection.
[f] Meristem-cultured healthy potato plants inoculated with PLRV were observed before the appearance of potato leafroll symptoms.

meristem-cultured healthy potato plants fixed with FAA or boiling tap water showed weak fluorescence reaction, while there was no fluoresence in those immediately observed without fixation or fixed with 5% glutaraldehyde. Considering these results, glutaraldehyde is thought to be the best of all fixation method used in this study.

The PLRV-infected potato plants showed strong fluorescence regardless of the staining methods tested. The meristem-cultured healthy potato plants stained with resorcin or observed by the DFD method of Namba *et al.* (17) showed no fluorescence. However, remarkable yellowish green-glistening fluorescence, resulting from aniline blue staining, was observed in all the tested plants, regardless of disease severity. Particularly, in the case of healthy-looking potato plants, most of the plants tested showed positive fluorescence reaction by aniline blue staining; very few of them showed slight fluorescence reaction by resorcin blue staining or by the DFD method (Table 5). Furthermore,

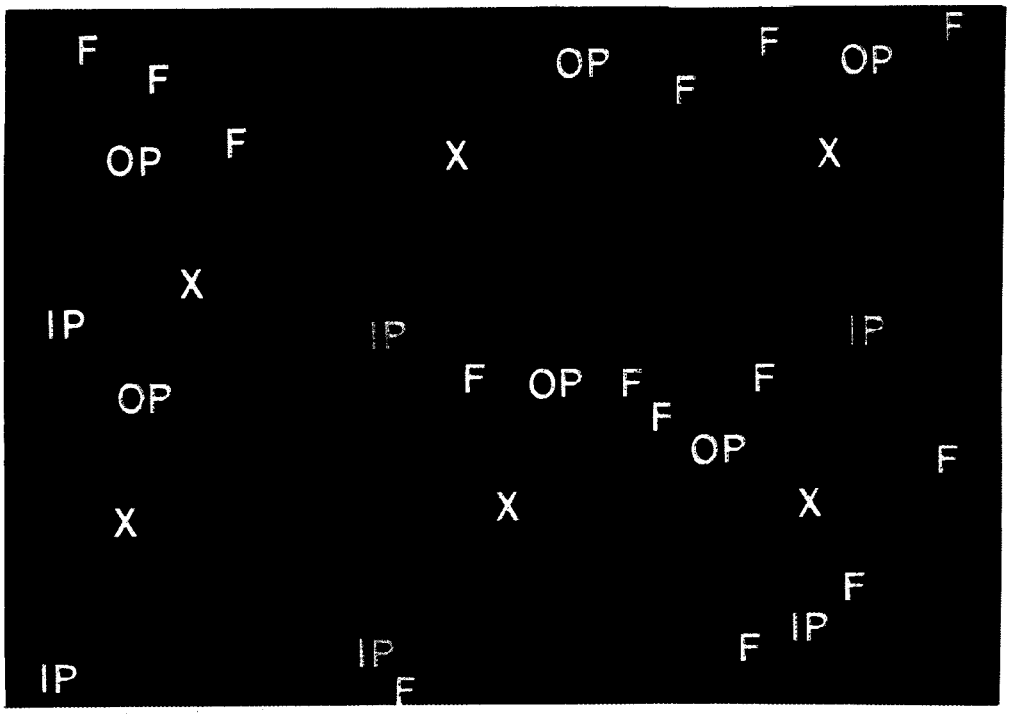

Fig. 1. Specific fluorescence of potato plants. A), C), E) PLRV-infected potato plant stained with resorcin blue, observed by DFD method (Namba *et al.*, 1982) and stained with aniline blue, respectively. B), D), F) Meristem-cultured healthy one treated as before. X : xylem, OP : outer phloem, IP : inner phloem, F : fluorescence.

Table 6. Comparison of diagnostic efficacy of resorcin blue stained callose-specific fluorescence to ELISA

Plant status	Diagnosis[a]	
	RBSF	ELISA
Healthy-looking[b]	7/25	2/6
PLRV-diseased[b]	36/49	24/32
PLRV-infected[c]	7/8	8/8
Meristem-cultured healthy	0/20	0/6

[a] No. of plants detected positively/no. of plants tested.
[b] Indexted through visual inspection.
[c] Meristem-cultured healthy potato plants inoculated with PLRV were observed before the appearance of potato leafroll symptoms.

deep blue fluorescence, resulting from resorcin blue staining, was more easily detectable than autofluorescence by the DFD method, as shown in Fig. 1.

Diagnosis of potato leafroll disease by fluorescence specific to resorcin blue-stained callose seemed to be correspondent to ELISA. To examine diagnostic efficacy for potato leafroll disease by the detection of the fluorescence specific to resorcin blue-stained callose, its reliability was compared to ELISA. The fluorescence in the phloem tissues of the PLRV-infected potato plant increased with the disease severity of potato leafroll (Table 6). Furthermore, most of the PLRV-infected potato plants with no definite leafroll symptoms showed fluorescence specific to resorcin blue-stained callose, which were turned out to be PLRV-infected by ELISA.

DISCUSSION

Since Igel and Lange (12) reported the diagnosis of potato viral diseases by callose test in 1955, callose substance has been generally thought to be a valuable indicator for rough separation of healthy and diseased potato tuber in seed potato production (4, 5). Particularly, abnormal formation of callose in the sieve area was accepted to be a direct result of potato leafroll virus infection (22, 24). We revealed that deep blue fluorescence was observed only in the PLRV-infected potato plants, which was consistent with the report of de Bokx (4) that only PLRV induced the abnormal quantities of callose in sieve area, but not consistent with the reports of Weichelt (24) and Igel and

Lange (12) that potato virus A induces abnormal callose and the callose test may be also used for the detection of other potato viruses. Hecht and Arenz (11) also revealed that potato virus A, X, and S do not induced abnormal callose.

Many works to improve the diagnostic reliability of potato leafroll disease by callose test have been extensively carried out. One of the representatives is searching for callose-specific staining reagents. Faulkner *et al.* (9), through the screening of fluorochromes for identification of several types of glucans, revealed that only β (1-3) glucan was autofluorescent and aniline blue staining lacked specificity for linkage configuration (α and β) or for linkage group, although the fluorescence of β (1-3) glucan was more intense than any other types of glucans. Shimomura and Dijkstra (19) reported that a bright yellow fluorescene by aniline blue staining could not be detected in the preparation of β (1-3) glucan treated with β (1-3) glucan hydrolase. The present study revealed that the resorcin blue staining is specific to callose in the PLRV-infected potato plants. However, as shown in Table 3, aniline blue staining showed positive fluorescence reaction in all the plant tissues tested, which means that aniline blue does not have the specificity to callose induced by PLRV infection and seems to be due to the structural varieties of callose. Consequently, it is thought that resorcin blue-stained callose specific fluorescence is more easily detectable than autofluorescence of the DFD method because of its deep blue color (Fig. 1).

Namba *et al.* (17) reported that the best material for the observation of the autofluorescence of callose was the fresh tissues of the petiole. In our study, the conditions for detection of PLRV-induced callose have been optimized to obtain the best results: The best source material and the best fixing method for the detection of callose were found to be the lateral branch of lower leaves and 5% glutaraldehyde for 1 hr. This disagreement may be due to the differences in the potato cultivars used.

In order to investigate the reliability of the fluorescence resorcin blue-stained callose for the diagnosis of potato leafroll disease, its efficacy was compared to ELISA. Our work revealed that PLRV-infected potato plants showed corresponding increment of the fluorescence intensity as absorption value in ELISA increased (Table 6). Considering the report of Wu and Dimitman (26) that the accumulation of callose is related to the viral translocation and distribution in plant tissues, this technique may be used for the diagnosis of potato leafroll disease with high reliability. Resorcin blue-stained callose specific fluorescence is thought to be able to overcome problems such as disease-masking or non-virus leafroll in visual inspection of potato leafroll disease and cut down the high cost in ELISA. However, a disadvantage of this method is that small quantities of callose may be present in healthy tubers (4) and for that reason it may be difficult to decide in every case whether the plant is infected or not. In the future, more reliable criteria for measuring the fluorescence objectively, for example the quantification of callose by fluorimeter for the exclusion of personal judgement of an operator, should be determined.

요 약

Resorcin blue로 염색한 진청색의 callose 특이적 형광은 공시한 PVX, PVY, PVS, PVM 및 PLRV 감염 감자 중에서 PLRV에 감염된 식물의 사부에서만 관찰되었다. Aniline blue로 염색한 식물체는 PLRV 감염 여부에 관계없이 비특이적 형광을 나타내어 감자 잎말림병의 진단에는 부적합한 것으로 생각되며 직접 형광 검출 기법(DFD)은 callose의 자체 형광에 의존하므로 식별에 있어 resorcin blue의 진청색 형광보다 효율성이 떨어지는 것으로 판단된다. PLRV 감염 유래의 특이적 형광 관찰용 시료의 부위 비교 실험 결과, 최적 부위는 하엽의 측지로 판명되었다. 이 실험에서 사용한 기법의 효소결합 면역항체 기법(ELISA)과의 진단 효율성 비교 실험에서 ELISA의 흡광도 값이 증가함에 따라 감염 식물내의 resorcin blue에 의한 진청색 형광 반응도 강해지는 것으로 나타났다. 앞으로 감염 식물 사부 요소내의 형광 반응을 정량적으로 측정할 수 있는 방법이 실용화를 위해 개발되어야 할 것으로 생각된다.

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REFERENCES

1. Cheadle, V. J., Gifford, Jr. E. M. and Esau, K. 1953. A staining combination for phloem and contiguous

tissues. *Stain Tech.* 28 : 49-53.
2. Clark, M. F. 1981. Immunosorbent assay in plant pathology. *Ann. Rev. Phytopathol.* 19 : 83-106.
3. Currier, H. B. 1957. Callose substance in plant cells. *Amer. Potato J.* 44 : 478-488.
4. De Bokx, J. A. 1967. The callose test for the detection of leafroll virus in potato tubers. *Eur. Potato J.* 10(3) : 221-234.
5. De Bokx, J. A. 1972. *Viruses of potatoes and seed potato production.* Centre for Agricultural Publishing and Documentation, Wageningen, the Netherlands, 233 pp.
6. Eschrich, W. 1956. Kallose (Ein kritischer Sammelbericht). *Protoplasma* 47 : 487-530.
7. Evert, R. F. and Derr, W. F. 1964. Callose substance in sieve elements. *Amer. J. Bot.* 51(5) : 552-559.
8. Evert, R. F. 1977. Phloem structure and histochemistry. *Ann. Rev. Plant Physiol.* 28 : 199-222.
9. Faulkner, G., Kimmins, W. C. and Brown, R. C. 1973. The use of fluorochromes for identification of β (1-3) glucans. *Can. J. Bot.* 51 : 1503-1504.
10. Gugerli, P. 1980. Potato leafroll virus concentration in the vascular region of potato tubers examined by enzyme-linked immunosorbent assay (ELISA). *Potato Res.* 23 : 137-141.
11. Hecht, H. and Arenz, B. 1963. Was weiss man heute, nach einem Jahrzehnt praktischer Blattoroll virustestung uber die Kallose? *Bayer. landw. Jb.* 40 : 839-856.
12. Igel, M. and Lange, H. 1955. Verfahren zur Fruhdiagnose von Viruskrankheiten bei Pflanzen Patentanmeldung beim dt. Patentamt, 18.4.1953.
13. Keller, E. R. and Berces, S. 1966. Check-testing for virus Y and leafroll in seed potatoes with particular reference to methods of increasing precision with the A6-leaf test for virus Y. *Eur. Potato J.* 9(1) : 1-14.
14. Kojima, M., Chou, T. G. and Shikada, E. 1978. Rapid diagnosis of potato leafroll virus by immune electron microscopy. *Ann. Phytopath. Soc. Japan* 44 : 585-590.
15. La, Y. J., Franckowiak, J. D. and Jr. Brown, W. M. 1977. Seed potato certification in Korea. *Kor. J. Plant Prot.* 16(3) : 163-170.
16. McMorran, J. P. and Allen, T. C. 1983. Maintenance, symptoms and distribution of potato virus X, S, Y, A, M and leafroll in potato tissue culture plantlets. *Amer. Potato J.* 60 : 839-847.
17. Namba, S., Yamashita, S., Doi, Y. and Yora, K. 1981. Direct fluorescence detection method (DFD) for diagnosing yellow-type virus diseases and mycoplasma disease of plants. *Ann. Phytopath. Soc. Japan* 47 : 258-263.
18. Schneider, I. R. 1965. Introduction, translocation and distribution of viruses in plants. *Adv. Virus Res.* 11 : 163-221.
19. Shimomura, T. and Dijkstra, J. 1975. The occurrence of callose during the process of local lesion formation. *Neth. J. Plant Pathol.* 81 : 107-121.
20. Smith, F. D. and Banttari, E. E. 1987. Dot-ELISA on nitrocellulose membranes for detection of potato leafroll virus. *Plant Disease* 71 : 795-799.
21. Smith, O. P., Harris, K. F., Toler, R. W. and Summers, M. D. 1988. Molecular cloning of potato leafroll virus complementary DNA. *Phytopathology* 78 : 1060-1066.
22. Styszko, R. 1983. Diagnosis of potato leafroll virus by the resorcin test(abstr.). *Zesyty Problemwe Postepow Nauk Rolniczyoh* 291 : 305.
23. Tamada, T. and Harrison, B. D. 1980. Factors affecting the detection of potato leafroll virus in potato foliage by enzyme-linked immunosorbent assay. *Ann. Appl. Biol.* 95(2) : 209-219.
24. Weichelt, Th. 1977. Improved production of "Resoblue" to indicate callose in potatoes. *Phytopath. Z.* 92 : 313-320.
25. Weidemann, H. L. 1981. Detection of potato viruses by the immuno-fluorescence technique. *Potato Res.* 24 : 255-266.
26. Wu, J. H. and Dimitman, J. F. 1970. Leaf structure and callose formation as determinants of TMV movement in bean leaves as revealed by UV irradiation studies. *Virology* 40 : 820-827.