PATHOLOGIE VÉGÉTALE

Phyllody of faba bean in the Sudan. II. — Detection of the disease by light microscopy : fluorescence, bright field, interference contrast and observation of MLOs by transmission electron microscopy

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SUMMARY

Several techniques of fluorescence, light and electron microscopy were used to detect MLOs in sieve tube elements of phyllody diseased faba bean plants. The DNA-binding fluorochrome Bisbenzimid H 33258 provided satisfactory results when used with sections prepared from fresh materials using freezing microtome or cryostat and with fixed materials embedded in paraffin or historesin. While sections from fresh materials provided rapid means of indexing, historesin and paraffin have the advantage of better preservation of anatomical characteristics of plant tissues. This was particularly the case of historesin since sections were processed without removing the embedding medium. Semi-thin sections (250-350 nm) prepared from epoxy resin-embedded plant tissue are not amenable to staining with DNA-specific fluorochromes, hence they could not be used for fluorescence microscopy. Alternatively, 2 methods of ordinary light microscopy could be utilized with such sections. The first was to examine unstained sections in a light microscope with differential interference contrast of Nomarski, and the second implied staining with thionin and acridine orange and observation in a bright field microscope. Both techniques, though non-specific to MLO provided additional tool in cytopathological studies of diseased plants and could be useful in determining areas meriting further examination in electron microscopy. The presence of MLOs in plant tissues, used for light and fluorescence microscopy, was confirmed in ultrathin and semi-thin sections observed by transmission electron microscopy.

Additional key words : *Bisbenzimid H.*

RÉSUMÉ

Phyllodie de la fève au Soudan. II — Détection de la maladie par la microscopie photonique : fluorescence, lumière normale, contraste interférentiel et mise en évidence des MLOs en microscopie électronique par transmission.

Plusieurs techniques de microscopie photonique en fluorescence et en lumière normale ainsi que de microscopie électronique ont été utilisées pour détecter les MLO à l'intérieur des tubes criblés de la fève atteinte de phyllodie. Un fluorochrome spécifique de l'ADN, Bisbenzimid H 33258 ou réactif de Hoechst, a donné des résultats satisfaisants quel que soit le mode de préparation du matériel végétal : tissu frais coupé au cryostat, tissu fixé et inclus dans la paraffine ou dans l'historésine. Les coupes préparées à partir de matériel frais ont permis l'indexage rapide de l'infection alors que l'inclusion dans la paraffine et surtout dans l'historésine a l'avantage de faciliter la caractérisation des structures anatomiques des tissus. L'historésine présente un intérêt particulier car les coupes peuvent être colorées en présence du milieu d'inclusion.

De plus, des coupes semi-fines de 250 à 350 nm d'épaisseur ont été préparées à partir de tissus fixés et inclus dans une résine époxy. Elles ne présentent pas de fluorescence. Aussi, 2 méthodes de microscopie photonique en lumière normale ont été alternativement employées : la première consiste en l'observation des coupes non colorées en contraste interférentiel de Nomarski et, la deuxième utilise la coloration par la thionine et l'acridine orange. Ces 2 techniques, bien que non spécifiques, facilitent la localisation des zones qui nécessitent une étude ultérieure en microscopie électronique. La présence des MLO à l'intérieur des tubes criblés des tissus malades a été confirmée sur les coupes ultrafines observées en microscopie électronique par transmission.

Mots clés additionnels : *Bisbenzimid H.*

I. INTRODUCTION

The association of MLOs with the faba bean phyllody disease was first reported in Morocco (COUSIN *et al.*, 1970) and recently in Sudan (JONES *et al.*, 1984; DAFALLA & COUSIN, 1986). These reports, largely based on electron microscopy, were confirmed through graft and dodder transmission of the disease agent to faba bean and several other susceptible hosts (DAFALLA & COUSIN, 1988). Such studies have established the etiological relationships of MLO with the disease but did not provide simple techniques for their routine detection. The need for such a technique thus emerged as the impact of the disease is increasingly becoming important.

Indirect detection of MLOs by using aniline blue, which stains excess callose, has largely been replaced by the use of DNA-binding fluorochromes such as DAPI and Bisbenzimid H (RUSSEL *et al.*, 1975). These latter have proved sensitive, rapid and adaptable to a variety of plant species (SEEMÜLLER, 1976; DOSBA & LANSAC, 1982; COUSIN & JOUY, 1984; SHARMA *et al.*, 1986). The technique was sensitive when used in sections of fresh material prepared in cryostat (SEEMÜLLER, 1976; SCHAPER & CONVERSE, 1985), or of preserved materials in paraffin (COUSIN & JOUY, 1984), and 2-hydroxyethyl methacrylate (GMA) (GRUNEWALDT-STÖCKER, 1985). Other techniques of light microscopy have made use of semi-thin sections of epoxy resin-embedded materials stained with thionin and acridine orange (SIEVERS, 1971; PAUL, 1980). These are now increasingly becoming useful in cytological studies of MLO-affected plants (COUSIN *et al.*, 1986).

In this study we evaluated the applicability of several techniques of fluorescence and ordinary light microscopy for the detection of MLOs in faba bean. Furthermore, we investigated a new method based on direct examination of unstained semi-thin sections in interference contrast microscopy. Parallel electron microscope observations were also made.

II. MATERIALS AND METHODS

A. Plant materials

Naturally diseased and experimentally infected faba bean plants with phyllody symptoms were used in these tests. Original source of the phyllody isolate was the central region of Sudan, which we believe to be identical to other phyllody isolates previously described in Khartoum province and Northern region of the country (NOUR, 1962).

B. Fluorescence microscopy

Pieces, 5 mm long, prepared from leaf mainveins, flower pedicels and young stem tissues from diseased and healthy plants were fixed in 4 % glutaraldehyde in 0.2 M cacodylate buffer pH 7.4 for 3-4 h, then washed in running tap water overnight. Fixed specimens were rinsed 3 times in 0.1 M cacodylate buffer, then grouped into three samples, each treated separately. The first was used directly for cryostat and freezing microtome. Sections 7-10 µm thick obtained on cryostat and of 30 µm thick obtained on freezing microtome were collected on glass slides, stained with Bisbenzimid H ($1 \mu\text{g} \cdot \text{ml}^{-1}$) for 20 min, washed with buffer and mounted in 1:1 (v/v) glycerol-buffer solution. The second group was processed for embedding in historesin (LKB-Produkter AB, Bromma, Sweden). Fixed samples were dehydrated in a series of graded ethanol (70 %-95 % ethanol), then infiltrated for 2 h in 1:1 (v/v) mixture of 95 % ethanol and infiltration solution (glycolmethacrylate without hardener). They were then transferred to pure infiltration solution for infiltration overnight and finally embedded in the same solution after adding a hardener. Longitudinal and cross sections 2-3 µm thick were prepared and stained as described previously. The third group was processed and embedded in paraffin according to COUSIN & JOUY (1984). Deparaffinized 7-8 µm-thick sections obtained were also stained and

Figure 1

Longitudinal section (7 Óm) through the phloem region of flower pedicel from diseased faba bean plant. Samples fixed, paraffin embedded, deparaffinized and stained with HOECHST's reagent. Sieve tubes (st) containing fluorescent MLOs more concentrated toward sieve plates (sp). Fluorescent nucleus. (N) ($\times 560$)

Coupe longitudinale (7 Óm) de tissu libérien de pédoncule floral de fève infectée. Echantillons fixés, inclus dans la paraffine, déparaffinés et colorés par le réactif de HOECHST. Tubes criblés (st) contenant des MLOs plus concentrés vers les parois criblées (sp). Noyau fluorescent (N). ($\times 560$).

Figure 2

Transverse section (7 Óm) through the area corresponding to figure 1 showing fluorescent MLOs inside several sieve tubes (st). The fluorescence of nuclei (N) differs from that of MLOs. ($\times 560$).

Coupe transversale (7 Óm) de la zone correspondant à la figure 1 montrant des MLOs à l'intérieur de plusieurs tubes criblés (st). La fluorescence des noyaux (N) se distingue de celle des MLOs. ($\times 560$).

Figure 3

Longitudinal section (3 Óm) from historesin (glycol-methacrylate) embedded flower pedicel of diseased plant. Sieve tubes packed with MLOs, nucleus (N), sieve plate (sp). ($\times 560$).

Coupe longitudinale (3 Óm) de pédoncule floral de plante malade inclus en historésine (Glycol-méthacrylate). Tubes criblés remplis de MLOs, noyau (N), paroi criblée (sp). ($\times 560$).

mounted as described above. Observations were made in a Leitz-Dialux-20 universal epifluorescence microscope with an HBO-50-W mercury source and equipped with 2 filter sets for U.V. and blue light (SHARMA *et al.*, 1986). Stained sections from diseased and healthy plants were compared with each other and with autofluorescence of unstained counterparts.

With each technique, several plant parts, including sections from stems, leaf petioles and midribs, flower pedicels and roots, were examined. Both longitudinal and transverse sections were considered and attempts were made to make observations on consecutive serial sections.

C. Bright field and interference contrast microscopy

Small pieces of 1 mm length obtained from stems, leaf midribs and flower pedicels were processed and embedded in epoxy resin following a method described by COUSIN *et al.*, (1986). These pieces had been collected from proximity of the previous ones observed in fluorescence microscopy. Semi-thin sections (250-350 nm) were prepared from diseased and healthy plants using a Reichert OM U2 ultramicrotome. They were collected on glass slides with drops of 0.025 % agar in distilled water, carefully dried and kept for use. A group of these sections was directly observed, without staining, in a Reichert Polyvar optical microscope equipped with a universal condenser for interference contrast.

The other group of sections was stained with thionin and acridine orange following methods of PAUL (1980) and COUSIN & JOUY (1984). They were then examined in ordinary bright field light microscope.

D. Transmission electron microscopy

Ultrathin (50-80 nm) and semi-thin (250-350 nm) sections prepared from epoxy resin embedded samples previously used in bright field and interference contrast microscopy were laid on copper grids, double stained with uranyl acetate and lead citrate and examined in a Philips EM 300 transmission electron microscope.

III. RESULTS

A. Fluorescence microscopy

Reactions were generally considered positive when fluorescent DNA material present in sieve tube elements

was regularly distributed in cases of low concentrations and fairly continuous in cases of high concentrations. In addition it should be clearly distinguished from fluorescent nuclei.

Positive fluorescence was recorded in most diseased samples regardless of plant part examined or method of specimen preparation. Fluorescent DNA material or presumed MLO particles were more frequently observed in functional sieve tubes determined according to criteria described by SCHAPER & SEEMÜLLER (1982). Various degrees of fluorescence intensity were observed and ranged from intermittent, indicating individual particles or small clusters, to continuous in case of sieve tubes packed with DNA material. These different intensities seem to be directly correlated with the degree of sieve tube invasion by MLOs. Thus, heavy colonization was observed in samples from young stems of plant tips and lateral sprouts, young leaf petioles and flower pedicels. Main stem tissues, petioles and midribs of older leaves, on the other hand, have shown lesser and varying degrees of light MLO colonization.

All techniques of specimen preparation have proved satisfactory for the indexing of fluorescence. Freezing microtomy provided preliminary indexing, although it was sometimes hard to differentiate nuclei since such sections are thick. The quality of fresh sections was highly improved in cryostat as they were sufficiently thin (7 µm) to permit differentiating nuclei from MLO clusters. The ease of cutting sections seemed to highly depend on plant material. Relatively woody portions were the best working material for obtaining good quality sections, while tender parts, especially useful for the detection of MLO colonization, were difficult to cut.

This difficulty could largely be overcome by using paraffin and historesin. The use of paraffin offered the advantage of obtaining ribbons of unlimited number of serial sections both in longitudinal and cross sections (fig. 1, 2). This facilitated systematic localization of MLOs in different sieve tube elements.

Historesin offered several advantages over cryostat and paraffin. Its application was less time consuming, economic and provided better preservation of plant tissues. Further, it permitted making sections of less than 3 µm allowing better visualization of MLOs and other plant cellular organelles (fig. 3). The only disadvantage was the difficulty of obtaining ribbons of serial sections.

B. Interference contrast microscopy

Observations made on unstained semi-thin sections from resin-embedded diseased plant material examined

Figure 4

Unstained semi-thin section (250 nm) through the phloem of flower pedicel of diseased plant, examined by NOMARSKI's interference contrast microscopy. Sieve tube (st) containing MLOs (M), sieve plate (sp), nucleus (N), xylem (X). (× 800).

Coupe semi-fine (250 nm) non colorée dans le phloème de pédoncule floral de plante malade, observée au microscope photonique à contraste interférentiel de NOMARSKI. Tube criblé (st) contenant des MLOs (M), paroi criblée (sp), noyau (N), xylème (X). (× 800).

Figure 5

Semi-thin section stained with thionin and acridine orange, observed in phase contrast microscopy. MLO (M), sieve tube (st), sieve plate (sp), nucleus (N), xylem (X). (× 350).

Coupe semi-fine colorée avec la thionine et l'acridine orange et observée au microscope à contraste de phase. MLO (M), tube criblé (st), paroi criblée (sp), noyau (N), xylème (X). (× 350).

by interference contrast microscopy showed the presence of amorphous particles in phloem sieve tubes. These particles appeared as true structural bodies on section surface and corresponded in size and shape to MLO particles (fig. 4). The major advantage of this method was that it provided a three-dimensional image of MLOs and cellular organelles.

C. Bright field microscopy

Sections stained with thionin and acridine orange and examined by bright field microscopy confirmed the presence of MLO particles in sieve tubes of diseased plants (fig. 5). They stained light grey and correspond in size to those seen before. Other cellular organelles such as nuclei could easily be distinguished (fig. 5). The technique could thus be utilized to locate areas for examination in electron microscopy.

D. Transmission electron microscopy

Both ultrathin and semi-thin sections through phloem tissues of diseased plants examined by electron microscopy have confirmed the presence of MLO particles (fig. 6). They possessed a three layered unit membrane (inset) characteristic of Mollicutes, ribosomes and DNA. Observations on semi-thin sections revealed the absence of helical forms. These results confirmed those from bright field and interference contrast microscopy obtained from the same epoxy resin embedded samples. This also confirmed fluorescence microscopy as the samples had been collected from close proximity of the previous ones.

IV. DISCUSSION

The staining technique using the DNA-specific fluorochrome Bisbenzimid H 33258 proved satisfactory for detecting MLOs in phyllody diseased faba bean plants. Fluorescent MLO particles were detected in sieve tube elements from almost all plant parts. The technique was also useful for evaluation of the extent of MLO invasion in various plant tissues. Individual MLO particles were readily detectable when sieve tubes were lightly colonized, and intense fluorescence throughout sieve tube lumens was recorded in case of heavy invasion. Various

grades of fluorescence intensity may thus be used quantitatively to rate MLO invasion in plant tissues concerned.

All methods used for specimen preparation have proved satisfactory. Freezing microtomy is rapid and easy to perform but provides only preliminary indexing and its use is limited to the hard woody plant parts which contain less MLO. Sections prepared in cryostat were so thin that one-cell-thick sections could be obtained. This allows better visualization of fluorescent MLO particles and better localization of them in sieve tube elements. Both techniques seem to be suited for routine indexing especially if many samples are to be tested in a short period of time.

Embedding in paraffin and historesin seems to be more suited for detailed histopathological studies. Paraffin provides long preservation of tissues and permits cutting ribbons of several serial sections. Historesin permits cutting of sections of as thin as 2 μ m. In addition, it is less time consuming and better conserves anatomical characteristics of plant tissues since it is not removed from sections before staining as in the case of paraffin. Unlike epoxy resins, historesins (glycolmethacrylate) conserve DNA fluorescence when stained with specific fluorochrome. It would thus be interesting to develop embedding media based on this product, which have the sensitivity to fluorochromes used in fluorescence microscopy and are also hard enough to use in electron microscopy.

Light microscopy of semi-thin sections from epoxy resin embedded material, though it provided preliminary indications of MLOs, is still far less sensitive than fluorescence indexing. Thionin which stains MLOs is not specific and it stains also other cellular organelles. Acridine orange allows better visualization of cell walls and helps to determine different cells of phloem tissues. The combination of these 2 stains thus seem to provide useful indications of areas to be further studied in electron microscope. Nomarski Interference contrast gives general topographical aspects with three-dimensional image of sieve tube elements. The presence of MLOs was indicated by structural protrusions throughout sieve tubes.

The results of fluorescence and bright field or interference contrast microscopy were confirmed in electron microscopy. Among the different methods used, fluorescence indexing seems to be the most promising. Its use for routine detection of MLOs in faba bean in thus highly advocated.

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Figure 6

Longitudinal section through the phloem of flower pedicel from diseased faba bean. MLOs distributed on each side of the sieve plate (sp), sieve pore (spo), P protein (Pp). ($\times 42.000$).

Detail showing the tripartite unit membrane (). ($\times 126.000$).

Coupe longitudinale dans le phloème de pédoncule floral de fève malade. MLOs répartis de part et d'autre de la paroi criblée (sp), pore du crible (spo), P protéine (Pp). ($\times 42.000$).

Détail montrant la membrane unitaire tripartite () des MLOs. ($\times 126.000$).

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