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A comparative study of fluorescence in stems of Vinca rosea infected with mycoplasmas of different plant origins

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Abstract

A histochemical study with aniline blue staining revealed a positive correlation between the time of external symptom development and abnormal fluorescence in phloem tissue of *Vinca rosea* plants grafted with mycoplasma-infected *Vinca* scions. A range of abnormal fluorescence was observed in phloem tissue of *Vinca* stems after mycoplasmas of 15 different plant origins were respectively introduced by graft-inoculation. The degree of fluorescence intensity and distribution in phloem tissue was related to the severity of disease. Results from examination of mycoplasmas from culture did not suggest that these organisms themselves contribute to the abnormal fluorescence in phloem of infected *Vinca*.

Introduction

Many yellows-type or witches' broom diseases have been associated with mycoplasma-like organisms (hereafter called mycoplasma) since the first claim of Doi et al. in 1967. The mycoplasmas can be detected by electron microscopy usually in phloem cells of infected plants after ultrathin sectioning.

Plants infected with mycoplasmas show a variety of symptoms (Bos, 1970). However, common to most diseases of this group is the regular occurrence of pleomorphic mycoplasma bodies in, and their specific effects on the phloem.

Histological aberrations in phloem elements of witches' broom-diseased plants can be detected by fluorescence microscopy, using aniline blue as fluorochrome (Hiruki and Shukla, 1973). This method has proved effective also in locating cytological abnormalities, particularly callose deposition in phloem, in other herbaceous plants and young trees (Hiruki and Dijkstra, 1973b; Dijkstra and Hiruki, 1974).

This paper will report the results of a comparison of abnormal fluorescence in phloem of a number of *Vinca rosea* plants separately inoculated with mycoplasmas originating from 15 different plants.

Pure mycoplasma cultures were also examined to find out whether mycoplasmas themselves contribute to the abnormal fluorescence in infected plants.

Materials and methods

Vinca plants. Except sandal spike (Dijkstra and Lee, 1972) the disease samples were

selected from a collection of yellows diseases maintained in Station de Cytopathologie, St-Christol, France. The mycoplasmas, except those of *Vinca* yellows, were first transferred to *Vinca* plants by means of *Cuscuta subinclusa*. Further transmission in *Vinca* was by wedge-grafting. Descriptions of certain diseases included in this investigation appeared elsewhere (Dijkstra and Lee, 1972; Giannotti and Vago, 1971; Giannotti and Delattre, 1972; Giannotti et al., 1968, 1972; Marchoux et al., 1969, 1972; Morvan et al., 1973).

Fluorescence microscopy. Samples were taken from young internode regions, about 1.0–1.5 cm from the apex, of *Vinca* plants showing typical symptoms. Control samples were taken from uninoculated plants. All samples were obtained from potted plants immediately before examination. Free-hand sections, about 20 to 30 μm thick, were made within 5 to 10 min after-excising the stem. They were fixed immediately by boiling in tap water for 3 to 4 min and then stained in 0.01 % aniline blue in 1/15 M K_2HPO_4, pH 8.0 for 20 min. Stained sections from both uninoculated and inoculated plants were simultaneously mounted in a small amount of aniline blue solution on a glass slide.

Observations of approximately 10 sections per sample were made as described earlier (Hiruki and Shukla, 1973; Hiruki and Dijkstra, 1973b). The examination was repeated at least once using different stem tissue from the same plants before rating each sample for fluorescence.

Mycoplasma cultures. Mycoplasma cultures were maintained by Giannotti as described elsewhere (Giannotti and Vago, 1971; Giannotti et al., 1971, 1972; Giannotti and Delattre, 1972). The medium MI 71 was used to culture mycoplasma of tomato stolbur 'SM', and the medium MI 71U for those of cabbage yellows, clover phyllody-1 and lavender yellows. For light and electron microscopy, mycoplasmas in liquid medium were sedimented by centrifugation (Sorvall RC2-B, rotor ss-1) at 10,000 rpm for 20 min. Mycoplasma colonies on solid media were removed with a scalpel. A drop of aniline blue (0.01 % in 1/15 M K_2HPO_4, pH 8.0) was added to mycoplasmas on a glass slide before fluorescence examination. Negative stain employed was 2 % ammonium molybdate, pH 7.1 (Cole et al., 1973).

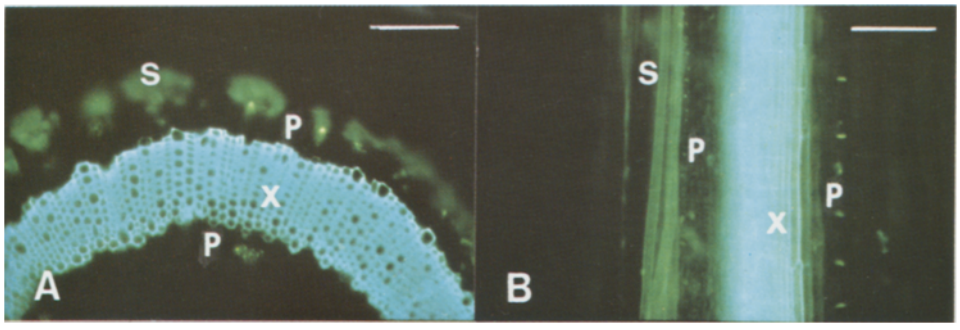
Results

External symptoms. Stem tissue samples were obtained from *Vinca* plants 2 to 3 months after graft-inoculation. Diseased *Vinca* plants showed a range of symptoms, depending on the host origin of mycoplasmas. Among them were chlorosis, elongation of internodes, greening, narrowing of leaves, phyllody, stunting, and yellows. These symptoms in infected plants often developed in combinations and were difficult in some cases to classify accurately.

Seven diseases showed relatively mild symptoms in *Vinca*: cabbage yellows, carrot yellows, clover phyllody-1, cotton phyllody, lavender yellows, rape yellows, and sandal spike (at St-Christol) (Table 1).

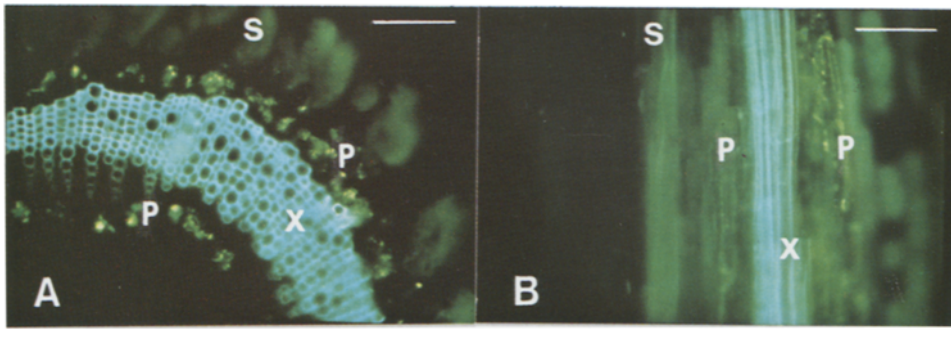
Seven diseases developed severe symptoms in *Vinca*: apple proliferation, apricot chlorotic leaf roll, *Plantago* yellows, sandal spike (at Wageningen), tomato stolbur 'C', tomato stolbur 'SM' and *Vinca* yellows (Table 1).

Plate 1. Sections of a healthy *Vinca rosea* stem. A, tranverse section; B, longitudinal section. A bar in each figure represents 100 μm. P, phloem; S, sclerenchyma; X, xylem. The film used was Agfa (CT 18).



Plaat 1. Coupes van een gezonde stengel van Vinca rosea. A, dwarsdoorsnede; B, lengtedoorsnede. De vergrotingsstreep in iedere figuur geeft 100 μm weer. P, floëem; S, sclerenchym; X, xyleem. De gebruikte film was Agfa (CT 18).

Plate 2. Sections of a *Vinca rosea* stem infected with sandal spike mycoplasma. A, transverse section; B, longitudinal section.



Plaat 2. Coupes van een stengel van Vinca rosea geïnfecteerd met mycoplasma van de 'spike'-ziekte van de sandelboom. A, dwarsdoorsnede; B, lengtedoorsnede.

Table 1. Symptoms on *Vinca rosea* and the results of fluorescence microscopy examination of sections of stem inoculated with mycoplasmas of different origins.

Original disease	Symptoms[1]	Fluorescence[2]
Apple proliferation	C, sS, sY	++
Apricot chlorotic leaf roll	G, → Y, S	+ → ++
Cabbage yellows	C, S, Y, y	+
Carrot yellows	C, P, S, Y	+
Clover phyllody-1	C, P, S, Y	+
Cotton phyllody	C, P, S, Y	+
Croton witches' broom	G, S	±
Lavender yellows	C, P, S, Y	+
Plantago yellows	C, Y, S	+ → ++
Rape yellows	C, S, Y, y	+
Sandal spike	C, P, S, Y (at St. Christol)	+
	C, G, P, sS, Y (at Wageningen)	+
Tomato stolbur 'C'	C, sS, sY	++
Tomato stolbur 'SM'	C, sS, sY	++
Tomato stolbur 'P'	E, G	—
Vinca yellows	C, sS, sY	++

[1] C, chlorosis; E, elongation of internodes; G,greening; P, phyllody; S, stunting; Y, yellows; y, discoloration of young stem, petiole and veins; s, severe.
[2] Visual rating of fluorescence intensity and distribution in phloem elements: –, normal; ±, nearly normal; +, abnormal; ++, very abnormal.

Tabel 1. Symptomen op Vinca rosea *en de resultaten van fluorescentiemicroscopisch onderzoek van stengelcoupes van deze planten na inoculatie met mycoplasma's van verschillende herkomst.*

Abnormal fluorescence and mycoplasma origin. After staining with aniline blue, stem sections from healthy *Vinca* plants showed three kinds of UV fluorescence: sclerenchyma cells were pale yellowish blue, sieve elements of the primary phloem (external and internal) brilliant yellow, and xylem cells pale blue (Plate 1A and B). Only xylem cells had autofluorescence. In infected stems pale to bright yellow fluorescence also was observed in cells of the rather extensively developed secondary phloem (external and internal) (Plate 2A and B).

The seven diseases with relatively mild symptoms in *Vinca* at St-Christol, as well as sandal spike at Wageningen, were associated with abnormal fluorescence in phloem elements (Table 1, Plate 2A and B, Fig. 1A). This abnormality always started in the external phloem region and then spread to the internal phloem.

With six out of the seven diseases that developed severe symptoms (sandal spike at Wageningen being the exception) (Table 1) intense fluorescence was observed in both external and internal phloem, and abnormal development of secondary phloem tissue (external and internal) was evident (Fig. 1B, 2A and B).

Mycoplasma associated with croton witches' broom produced only slightly abnormal fluorescence in the primary phloem region of infected plants (Table 1). Tomato stolbur 'P' was unique in that elongation of internodes and greening were its main symptoms, and there was no excessive fluorescence (Table 1).

Fig. 1. Sections of *Vinca rosea* stems infected with clover phyllody-1 mycoplasma (A) and with tomato stolbur 'SM' (B).

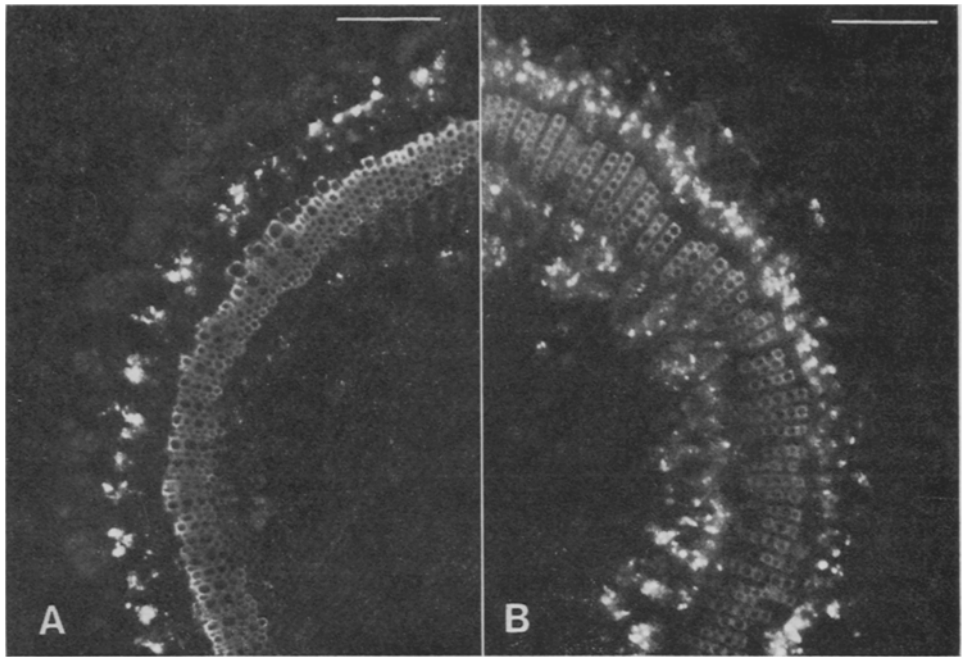


Fig. 1. Coupes van stengels van Vinca rosea geïnfecteerd met de mycoplasma's van klaver-fyllodie-1 (A) en stolbur 'SM' van tomaat (B).

Fig. 2. Sections of *Vinca rosea* stems infected with stolbur 'C'. A, transverse section; B, longitudinal section.

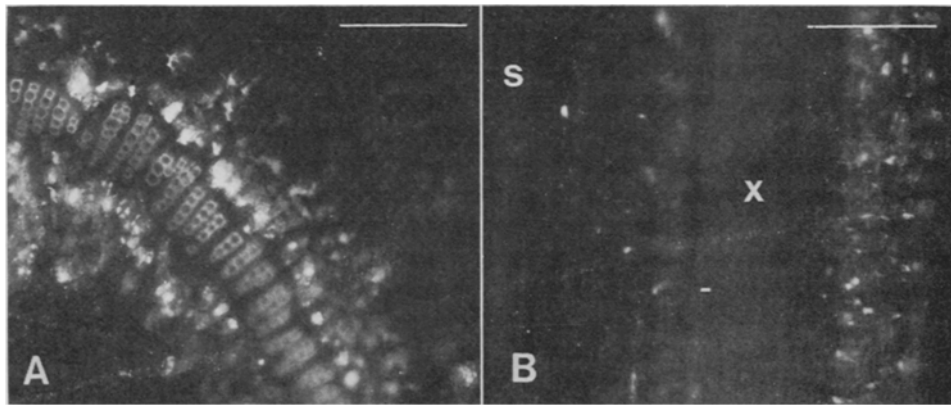


Fig. 2. Coupes van stengels van Vinca rosea geïnfecteerd met stolbur 'C' van tomaat. A, dwarsdoorsnede; B, lengtedoorsnede.

Time of appearance of abnormal fluorescence. Two sets of *Vinca* plants, separately graft-inoculated with apple proliferation or clover phyllody-1 mycoplasma, were examined to determine the time of appearance of abnormal fluorescence due to callose deposition in phloem cells, and to relate fluorescence to the severity of symptoms developed in newly grown shoots. After recording the symptoms developed in the side shoots, samples were taken at certain time intervals and studied for fluorescence (Table 2). There was a general agreement between the development of external symptoms and intensity and distribution of fluorescence in phloem elements.

Slightly abnormal fluorescence was detected in a *Vinca* plant that had been in graft contact with apple proliferation mycoplasma for 14 days but did not show any external symptoms. This plant later developed severe symptoms as well as very abnormal fluorescence. *Vinca* plants showed clear external symptoms and abnormal fluorescence 26 days after graft-inoculation with apple proliferation mycoplasma, and 28 days with clover phyllody-1 mycoplasma. The intensity and distribution of fluorescence in the secondary phloem region increased as external symptoms fully developed.

Table 2. Symptom development and fluorescence rating in phloem cells of *Vinca rosea* inoculated with mycoplasmas of clover phyllody-1 and of apple proliferation, respectively.

Apple proliferation				Clover phyllody-1			
number of days after grafting	symptoms[1,2]		fluorescence[3]	number of days after grafting	symptoms[1,2]		fluorescence[3]
14	0/3	A	−	12	0/5	A	−
		A	−			A	−
		A	±			A	−
						A	−
Control	0/3	A	−			A	−
				Control	0/5	A	−
26	2/3	A	±	28	2/5	A	−
		B	+			A	±
		B	+			B	+
						A	−
Control	0/3	A	−			B	+
				Control	0/5	A	−
78	3/3	C	+	74	4/5	B	+
		D	++			B	+
		D	++			C	+
						A	−
Control	0/3	A	−			C	+
				Control	0/5	A	−

[1] Number of plants apparently diseased over number of plants grafted.
[2] Rating of symptoms developed in newly grown shoots: A, no apparent symptoms; B, vein-clearing and mild leaf curling; C, pronounced yellowing; D, severe stunting and shortening of internodes.
[3] Rating of fluorescence intensity and distribution in phloem elements: −, normal; ±, slightly abnormal; +, abnormal; ++, very abnormal.

Tabel 2. De ontwikkeling van de symptomen en de mate van fluorescentie in floëemcellen van Vinca rosea *geïnoculeerd met mycoplasma's van klaver-fyllodie-1 en appel-proliferatie.*

Fluorescence of mycoplasma from culture. The mycoplasmas of cabbage yellows, clover phyllody-1, lavender yellows and tomato stolbur 'SM', obtained from liquid and/or solid culture media, had faint blue autofluorescence. However, the intensity of fluorescence did not change after adding a solution of aniline blue. The mycoplasma bodies were morphologically intact when observed in the electron microscope following negative staining.

Discussion

In this investigation a positive correlation was found between accumulation of fluorescent material and development of external symptoms in recently grafted plants. Host plants need at least 3 to 4 weeks to accumulate detectable amounts of fluorescent material after grafting. It is interesting that slightly abnormal fluorescence was detected in a plant that had been in contact with apple proliferation-diseased tissue for 14 days only and that did not show external symptoms. Later, the plant developed severe symptoms as well as very abnormal fluorescence.

Also interesting was the exception that tomato stolbur 'P', which stimulated elongation of internodes, did not cause extra fluorescence. Marchoux et al. (1969) reported that the 'P' isolate differed from other stolbur isolates such as 'C', 'M', and 'SM' in not causing a swift wilting in diseased egg-plants of certain varieties. Whether this is related to the amount of mycoplasmas in phloem cells or to low virulence remains to be determined. Stolbur isolates 'C' and 'SM' were quite effective in stimulating fluorescence in phloem cells (Table 1, Fig. 1 and 2).

Fluorescence microscopy has been used earlier to detect internal lesions in plants affected by grape vine 'flavescence dorée' disease (Carle, 1965), yellows (Cousin and Grison, 1966; Cousin, 1967) and stolbur (Cousin et al., 1968). These authors observed a strong fluorescence in internal lesions in absence of fluorochrome but they did not find callose as a fluorescent material. On the other hand, abundant callose deposition, as detected by lacmoid staining, was reported in the replacement phloem cells of plants with pear decline (Batjer and Schneider, 1960), now associated with mycoplasmas (Hibino and Schneider, 1970). We have earlier observed fluorescence in the secondary phloem cells with abundant intact mycoplasma bodies and we attributed it to abnormally accumulated callose in the affected phloem cell walls (Hiruki and Dijkstra, 1973a and b; Dijkstra and Hiruki, 1974).

Our experiments with mycoplasma cultures failed to show that mycoplasmas contain material(s) that fluoresces after aniline blue staining, although further investigations with various mycoplasma cultures are needed.

Samenvatting

Een vergelijkende studie van fluorescentie in stengels van Vinca rosea geïnfecteerd met mycoplasma's afkomstig van verschillende planten

Door histochemisch onderzoek, waarbij gekleurd werd met anilineblauw, is aangetoond dat er een verband bestaat tussen de intensiteit van fluorescentie alsmede de verdeling hiervan in het floëem en de hevigheid van de ziektesymptomen.

Gekleurde stengelcoupes van gezonde *Vinca*-planten vertoonden drie soorten ultra-

violet licht fluorescerende cellen: bleek-geelblauwe sclerenchymcellen, heldergele zeefelementen, behorende tot het primaire binnen- en buitenfloëem en bleekblauwe xyleemcellen (Plaat 1A en 1B). In stengels van geïnfecteerde planten kwam bovendien nog bleek- tot heldergele fluorescentie voor in de cellen van het sterk-ontwikkelde, secundaire binnen- en buitenfloëem (Plaat 2A en 2B).

Mycoplasma's van zeven ziekten, die betrekkelijk milde symptomen gaven op *Vinca*-planten in St. Christol, alsook die van de 'spike'-ziekte van de sandelboom met hevige symptomen op *Vinca* in Wageningen, veroorzaakten een fluorescentie in floëemelementen van deze planten die sterker was dan normaal (Tabel 1, Plaat 2A en 2B en Fig. 1A).

Mycoplasma's van zes van de zeven ziekten, die hevige symptomen deden ontstaan op *Vinca*-planten (de 'spike'-ziekte van de sandelboom op *Vinca* in Wageningen was de uitzondering) (Tabel 1), brachten in de elementen van zowel het binnen- als buitenfloëem sterke fluorescentie teweeg en bovendien een abnormaal-sterke ontwikkeling van het secundaire binnen- en buitenfloëem (Fig. 1B, 2A en 2B).

'Croton witches' broom' veroorzaakte slechts weinig extra fluorescentie in het primaire floëem van geïnfecteerde *Vinca*-planten. Stolbur 'P' van tomaat gaf in *Vinca* wel uitwendige ziekteverschijnselen (verlenging van de internodiën en vergroening van bloemen), doch geen abnormale fluorescentie (Tabel 1).

Het verband tussen het tijdstip van optreden van abnormale fluorescentie en de ontwikkeling van uitwendige symptomen is onderzocht (Tabel 2). Licht-abnormale fluorescentie werd aangetroffen in een *Vinca*-plant die 14 dagen lang door middel van een ent in contact was geweest met weefsel aangetast door appel-proliferatie; deze plant vertoonde echter (nog) geen uitwendige symptomen. Later ontwikkelden zich hevige uitwendige symptomen alsmede sterke fluorescentie. *Vinca*-planten vertoonden 26 dagen na enting met weefsel afkomstig van planten aangetast door appel-proliferatie duidelijk-zichtbare uitwendige symptomen alsook abnormale fluorescentie; in het geval van klaver-fyllodie was dit 28 dagen. De intensiteit en verdeling van de fluorescentie in het secundaire floëem namen toe naarmate de uitwendige symptomen zich verder ontwikkelden.

Mycoplasma-cultures (zowel in vloeibaar als vast medium), afkomstig van een aantal bovengenoemde mycoplasma-zieke planten, vertoonden een zwakke, blauwe autofluorescentie. De intensiteit van de fluorescentie veranderde niet na toevoeging van anilineblauw. We moeten daarom concluderen dat de sterke fluorescentie in floëemelementen van geïnfecteerde planten hoofdzakelijk moet worden toegeschreven aan de abnormaal-sterke callose-afzetting in de cellen en niet aan de aldaar aanwezige mycoplasma's zelf.

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References

Batjer, L. P. & Schneider, H., 1960. Relation of pear decline to rootstocks and sieve-tube necrosis. Proc. Am. Soc. hort. Sci. 76: 85–97.

Bos, L., 1970. Symptoms of virus diseases in plants. 2nd edition, pp. 206. Pudoc, Wageningen, the Netherlands.

Carle, P., 1965. Fluoroscopie des symptômes histologiques de la flavescence dorée de la vigne. Application à la détection rapide des lésions précoces sur cépage sensible (Baco 22A). Annls. Inst. natn. Rech. agron., Paris, Sér. C. (Annls. Epiphyt.) 16: 73–85.

Cole, R. M., Tully, J. G., Popkin, T. J., & Bové, J. M., 1973. Morphology, ultrastructure, and bacteriophage infection of the helical mycoplasma-like organism (*Spiroplasm citri* gen. nov., sp. nov.) cultured from 'Stubborn' disease of citrus. J. Bact. 115: 367–386.

Cousin, M. T., 1967. Histological study by means of fluorescence microscopy, of plants infected with 'yellow-type' viruses. In: C. Blatný (Ed.), Plant Virology. Proc. Conf. Czech. Pl. Virologists 6: 145–150. Olomouc 1967.

Cousin, M. T. & Grison, C., 1966. Premières observations concernant une fluorescence anormale dans le liber interne de plusieurs Solanées infectées par le virus du Stolbur et d'une Apocynacée atteinte de phyllodie. Annls. Inst. natn. Rech. agron., Paris, Sér. C (Annls. Épiphyt.) 17: 93–98.

Cousin, M. T., Grison, C. & Decharme, M., 1968. Étude comparée de plusieurs types de flétrissements de Solanacées. Polyphagie du stolbur. Annls. Inst. natn. Rech. agron., Paris, Sér. C (Annls. Épiphyt.) 19: 121–140.

Dijkstra, J. & Hiruki, C., 1974. A histochemical study on sandal (Santalum album) affected with spike disease and its diagnostic value. Neth. J. Pl. Path. 80: 37–47.

Dijkstra, J. & Lee, P. E., 1972. Transmission by dodder of sandal spike disease and the accompanying mycoplasma-like organisms via *Vinca rosea*. Neth. J. Pl. Path. 78: 218–224.

Doi, Y., Teranaka, M., Yora, K. & Asuyama, H., 1967. Mycoplasma or PLT group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows, or paulownia witches' broom. Ann. phytopath. Soc. Japan 33: 259–266.

Giannotti, J. & Delattre, R., 1972. Une nouvelle approche de l'étude épidémiologique d'une phyllodie, la virescence florale du cotonnier: Culture sélective de mycoplasmes extraits de quelques plantes et insectes homoptères de la biocénose. Coton Fibr. trop. 27: 1–7.

Giannotti, J., Morvan, G. & Vago, C., 1968. Micro-organismes de type mycoplasme dans les cellules libériennes de *Malus sylvestris* L. atteint de la maladie des proliférations. C. r. hebd. Séanc. Acad. Sci., Sér. D 267: 76–77.

Giannotti, J. & Vago, C., 1971. Rôle des mycoplasmes dans l'étiologie de la phyllodie du trèfle: culture et transmission expérimentale de la maladie. Physiol. vég., 9: 541–553.

Giannotti, J., Vago, C., Marchoux, G., Devauchelle, G. & Czarnecky, D., 1972. Caractérisation par la culture in vitro de souches de mycoplasmes correspondant à huit maladies différentes de plantes. C. r. Acad. Sci., D 274: 330–333.

Giannotti, J., Vago, C., Sassine, J. & Czarnecky, D., 1971. Culture et transmission de mycoplasmes phytopathogènes. C. r. hebd. Séanc. Acad. Sci., Sér. D 272: 1777–1778.

Hibino, H. & Schneider, H., 1970. Mycoplasma like bodies in sieve tubes of pear trees affected with pear decline. Phytopathology 60: 499–501.

Hiruki, C. & Dijkstra, J., 1973a. An anomalous form of mycoplasma-like bodies in periwinkle infected with the sandal spike agent. Neth. J. Pl. Path. 79: 112–121.

Hiruki, C. & Dijkstra, J., 1973b. Light and electron microscopy of *Vinca* plants infected with mycoplasma-like bodies of the sandal spike disease. Neth. J. Pl. Path. 79: 207–217.

Hiruki, C. & Shukla, P., 1973. Mycoplasmalike bodies associated with witches'-broom of bleeding heart. Phytopathology 63: 88–92.

Marchoux, G., Giannotti, J. & Laterrot, H., 1969. Le Stolbur P, une nouvelle maladie de type jaunisse de la tomate. Symptomes et examen cytologiques des tissue au microscope électronique. Annls. Phytopath. 633–640.

Marchoux, G., Leclant, F., Giannotti, J. & Cadilhac, B., 1972. Identification et épidémiologie des maladies à virus et à mycoplasmes infestant les Ombellifères dans le sud-est de la France. Actas III Congr. Un. fitopat. medit., Oeiras, 1972.

Morvan, G., Giannotti, J. & Marchoux, G., 1973. Untersuchungen über die Ätiologie des chlorotischen Blattrolls des Aprikosenbaums; Nachweis von Mykoplasmen. Phytopath. Z. 76: 33–38.

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