Susceptibility of Ten Red Clover (*Trifolium pratense*) Cultivars to Six Viruses after Artificial Inoculation

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Abstract

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Seedlings of *Trifolium pratense* L. cultivars were mechanically inoculated with Czech isolates of *Alfalfa mosaic virus* (AMV), *Clover yellow mosaic virus* (ClYMV), *Clover yellow vein virus* (ClYVV), *Red clover mottle virus* (RCMV), *White clover mosaic virus* (WClMV), and a newly discovered member of the *Cytorhabdovirus* genus. WClMV infected 75.4% of clover seedlings; cv. Rezista was the most susceptible (93.3%), while cv. Fresko was the least susceptible (58.3%). RCMV infected 59.6% of plants; the most susceptible was cv. Tempus (77.6%), the least susceptible cv. Sprint (38.3%). While WClMV infected a higher number of seedlings, RCMV revealed more severe symptoms on affected plants. On the basis of ELISA and RT-PCR results, no cultivar was susceptible to mechanical inoculation with ClYMV and cytorhabdovirus. Moreover, cvs Fresko and Sprint were not susceptible to ClYVV and AMV, respectively.

Keywords: *Red clover mottle virus*; *White clover mosaic virus*; DAS-ELISA; mechanical inoculation

Red clover (*Trifolium pratense*) is irreplaceable as a fodder crop in certain arable land. This does not pertain only to montane and submontane regions, as clover has been sown also in the lowlands of the temperate zones in Europe, North and South America, Asia, Australia, and New Zealand. A disadvantage of clover growing is its limited persistence, due in large measure to viral and phytoplasma diseases (Edwardson & Christie 1986; Taylor & Quesenberry 1996; Fránová *et al*. 2004; Jones 2012). There are 36 viruses listed to be able to infect red clover (Brunt *et al*. 1996) and doubtless the list could be extended with further discoveries (Fránová *et al.* 2004; Fránová & Jakešová 2012). However, only seven viruses were of widespread major importance: *Bean yellow mosaic virus* (BYMV), *Peanut stunt virus* (PSV), *Red clover vein mosaic virus (*RCVMV), *Pea streak virus (*PStrV), *Alfalfa mosaic virus (*AMV), *White clover mosaic virus (*WClMV), and *Clover yellow vein virus* (ClYVV) (Barnett & Diachun 1985). BYMV was the most prevalent virus infecting red clover in the southeastern United States (McLaughlin & Boykin, 1988) and probably was the most important worldwide (Smrtz *et al*. 1983). PSV was probably the second most prevalent virus of red clover at least in Kentucky (Naidu *et al*. 1995). Recently, red clover mottle virus (RCMV) has been recognised as the major pest for *Trifolium pratense* (EPPO 2013).

Breeding for resistance has been underway for many years. For example, in the United States, Kenstar and Arlington red clover cultivars have resistance to BYMV (Taylor & Quesenberry 1996). In the former Czechoslovakia, BYMV-resistant cultivars were found among 23 cultivars and new selections tested in the field and in a greenhouse (Pokorný 1989). There is BYMV-resistant tetraploid red clover cultivar Rezista, registered in the Czech Republic in 2005 under No. TFP06460. In the field, diploids showed lower values for death from infection and for mean intensity of infection. In Kentucy (USA), a red

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clover germplasm resistant to strain 204-1 of BYMV was released (Taylor *et al*. 1985). In Pennsylvania, 15 plants resistant to BYMV were selected from a mixed cultivar population of plants from 2-, 3-, and 4-year old fields and population of Kenstar red clover plants grown in a greenhouse (Sim *et al*. 1985). The selected plants survived repeated inoculations and were considered resistant to strain 204-1 of BYMV. In Hungary, phenotypic mass selection was used to artificially select for resistance to BYMV in a colchicine-induced tetraploid population (Halasz *et al*. 1985). The selected material showed field resistance while maintaining its level of green matter production. Inheritance of resistance to strains of BYMV has been investigated intensively, too (Broda & Fiedorow 1984; Taylor *et al*. 1986).

For WClMV, tolerance in red clover behaved as if it were controlled by polygenes (Martin 1989). Also, for RCVMV, resistance to a single isolate was controlled by a dominant gene (Khan *et al*. 1978).

AMV, *Cucumber mosaic virus* (CMV), BYMV, *Clover yellow mosaic virus* (ClYMV), ClYVV, RCMV, WClMV, new unnamed virus(es) of the genus *Cytorhabdovirus*, and new member of genus *Badnavirus* (named *Red clover bacilliform virus* – RCBV) were identified by DAS-ELISA and/or sequencing, observed by transmission electron microscopy and artificially transmitted to differential host plants in the latest screening for viruses in cultivated and wild growing red clover plants in the Czech Republic. The most frequent and economically important viruses seem to be RCMV and WClMV (Fránová *et al.* 2004, 2009, 2011; Fránová & Jakešová 2012). Symptoms of RCMV (mottle, mosaic, stunting) persist all over the vegetation period of red clover (Brunt *et al*. 1996; Valenta & Marcinka 1971), while symptoms of WClMV (mosaic, vein clearing, and mottle of different degrees of severity in various clover species) vary seasonally (Bercks 1971; Brunt *et al*. 1996). From the phytosanitary point of view, therefore, virus detection and identification are of highest importance. Although certain preventive measures may be taken, it is practically impossible to eliminate viral diseases of clover in the field. Here, we present a study on the susceptibility of ten red clover cultivars to virus isolates commonly present in legume crops in the climatic conditions of the Czech Republic.

MATERIAL AND METHODS

Seeds of ten red clover varieties were kindly provided by DLF-TRIFOLIUM Hladké Životice, s.r.o. (cvs Amos, Beskyd, Bivoj, Dolina, Fresko, Nodula, and Rezista – all 4*n*) and the Domoradice Breeding Station (cvs Sprint (4*n*), Start (2*n*), and Tempus (4*n*). *Alfalfa mosaic virus* (AMV, family: *Bromoviridae*, genus: *Alfamovirus*), *Clover yellow mosaic virus* (ClYMV, *Alphaflexiviridae*, *Potexvirus*), *Clover yellow vein virus* (ClYVV, *Potyviridae*, *Potyvirus*), *Red clover mottle virus* (RCMV, *Secoviridae*, *Comovirus*), *White clover mosaic virus* (WClMV, *Alphaflexiviridae*, *Potexvirus*), and a newly discovered member of the family *Rhabdoviridae* (genus *Cytorhabdovirus*) were used for mechanical inoculation of clover seedlings. Virus isolates had previously been transmitted mechanically from naturally infected red clover grown at various breeding stations in the Czech Republic, characterised, then maintained in differential host plants in an insect-proof greenhouse (Fránová *et al*. 2009, 2011). As sources of inocula, AMV, and *Cytorhabdovirus* were multiplied in *Physalis floridana* Rybd., ClYMV in *Gomphrena globosa* L., ClYVV in *Nicotiana occidentalis* Wheeler accession 37B, RCMV in *Pisum sativum* L., and *Phaseolus vulgaris* L. cv. Saxa, WClMV in *Phaseolus vulgaris* L.

Inoculum was prepared by homogenising symptomatic leaves of host plants in 0.1 mol/l of phosphate buffer (0.1 mol/l solution of $Na_2HPO_4 \cdot 12H_2O$, and 0.1 mol/l solution of $NaH_2PO_4 \cdot 2H_2O$), pH 7.0, with carborundum powder (silicon carbide, SiC) as abrasive. 1 g of fresh leaves was homogenised with 5 ml of buffer. Once the inoculum was prepared, the three first true leaves of clover seedlings were gently rubbed with sap using glass pestle on July 14, 2011. Each leaf of all three tiny leaves was rubbed all over the surface of the blades. About 60 plants of each examined cultivar were inoculated with a given virus and 10 plants of each cultivar were inoculated solely with the buffer and carborundum served as negative (healthy) controls. The seedlings were washed 2 h after inoculation and maintained outside under roof protected against rainfall. The average monthly temperature, rainfall, and hours of light per day were 17.3°C, 144.5 mm, and 162 h in July; 19.1°C, 71.8 mm, and 247 h in August; 15.3°C, 14.7 mm, and 218 h in September; 8.68°C, 29.8 mm, and 110 h in October; 2.47°C, 0.3 mm, and 121 h in November, respectively.

Symptoms were first evaluated one month after inoculation and subsequently over the course of the next three months. To examine the presence of viruses in both symptomatic and asymptomatic clover seedlings, DAS-ELISA kits (including commercially available positive controls) were used according to

the manufacturers' instructions (Bioreba AG, Reinach, Switzerland for AMV; Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany for ClYMV, ClYVV, WClMV; and Loewe Biochemica, Sauerlach, Germany for RCMV). Samples with extinction (E) higher than $x + 3$ SD (where: x – mean value; SD – standard deviation of the uninoculated controls) were scored as positive. The presence of cytorhabdovirus was determined by RT-PCR (Fránová *et al*. 2011, unpublished data). Leaves of healthy *N. occidentalis* plants and homogenisation buffer served as negative and blank controls, respectively. DAS-ELISA and RT-PCR were carried out from December 2011 to March 2012, i.e., during the 5th to 8th months after mechanical inoculation of clover seedlings.

RESULTS

The data summarised in Table 1 (the data of symptoms evaluation are from the 4th month after mechanical inoculation) indicate that red clover plants were most susceptible to WClMV infection. Out of 435 plants that were inoculated, 302 plants (69.4%) displayed virus-like symptoms, yet 328 plants (75.4%) were infected according to the ELISA. For WClMV, cv. Rezista (93.3%) was the most susceptible and cv. Fresko (58.3%) the least susceptible. Plants of all examined cultivars revealed weak or moderate systemic mosaic, vein clearing, mild curling (Figure 1), and dwarfing in later stages of infection.

RCMV also infected a high proportion of plants. Out of 591 plants that were inoculated, 310 plants (52.5%) displayed virus-like symptoms, yet 352 plants (59.6%) were infected according to the ELISA. Intensity of RCMV symptoms increased through four months' time from severe mosaic with variation of pale and dark sectors (mottling) to rings and spots, necrosis, smaller leaves and dwarfing. RCMV symptoms (severe mosaic, mottle, and leaf deformation) were clearly distinguishable from the mild systemic mosaic and vein clearing caused by WClMV infection (Figure 1). The cultivar most susceptible to RCMV infection was cv. Tempus, while seedlings of cv. Sprint were the least sensitive.

Examination of plants for presence of inoculated viruses using DAS-ELISA showed a positive reaction in all symptomatic plants (for all cultivars examined)

Table 1. Results of DAS-ELISA and symptom observation of *Trifolium pratense* cultivars mechanically inoculated with *Red clover mottle virus* (RCMV) and *White clover mosaic virus* (WClMV)

Virus	Cultivar	Number of plants			Symptoms
		inoculated	infected (ELISA)	symptomatic	
RCMV	Start	60	26 (43.3%)	23 (38.3%)	Mm, Lm, D
	Amos	60	38 (63.3%)	34 (56.7%)	Ms, Lm, D
	Beskyd	60	38 (63.3%)	32 (53.3%)	M, Lm, C, D
	Bivoj	56	39 (69.6%)	37 (66.1%)	M, Lm, D
	Dolina	60	31 (51.7%)	27 (45.0%)	M, Lm, D
	Fresko	59	28 (47.5%)	25 (42.4%)	M, Lm, D, +
	Nodula	58	42 (72.4%)	40 (69.0%)	M, Lm, D
	Rezista	60	42 (70.0%)	33 (55.0%)	Ms, Lm, D
	Sprint	60	23 (38.3%)	19 (31.7%)	M, Lm, D
	Tempus	58	45 (77.6%)	40 (69.0%)	M, Lm, C, D
Total		591	352 (59.6%)	310 (52.5%)	
WClMV	Start	60	46 (76.7%)	44 (73.3%)	Mm, C, D
	Amos	60	51 (85.0%)	47 (78.3%)	M, D
	Beskyd	60	45 (75.0%)	42 (70.0%)	Mm, C, D
	Bivoj	60	41 (68.3%)	36 (60.0%)	Mm, C, D
	Fresko	60	35 (58.3%)	32 (53.3%)	Mm, C, D
	Rezista	60	56 (93.3%)	55 (91.7%)	Mm, D
	Sprint	27	22 (81.5%)	20 (74.1%)	Mm, C, D
	Tempus	48	30 (62.5%)	26 (54.2%)	Mm, Lm, D
Total		435	328 (75.4%)	302 (69.4%)	

C – light curling; D – dwarfing in the late stage of infection; Lm – malformation of leaves; M – mosaic; Mm – mild mosaic; Ms – severe mosaic; + death of one plant

Figure 1. Symptoms on red clover cultivars infected with *White clover mosaic virus* (mild systemic mosaic and vein clearing) and *Red clover mottle virus* (severe mosaic, mottle and leaf deformation) 33 days after mechanical inoculation

inoculated with WClMV and RCMV but only rarely in asymptomatic plants (Table 1). The mean ELISA values (E_{405}) in positive clover samples reached 1.213 and 1.605 and in positive controls 1.563 and 2.020 for WClMV and RCMV, respectively, while healthy controls were equal to blank values. No virus-like symptoms were observed in clover seedlings inoculated with AMV, ClYMV, ClYVV, and cytorhabdovirus, yet some of them were ELISA-positive (AMV – cv. Start: 60 plants inoculated/2 plants positive, cv. Amos: 58/1, cv. Beskyd: 54/1, cv. Rezista: 60/2, cv. Tempus: 40/1; ClYVV – cv. Start: 60/1, cv. Amos: 60/2, cv. Beskyd: 60/1, cv. Bivoj: 53/1, cv. Nodula: 43/1, cv. Rezista: 60/1, cv. Sprint: 60/1). The mean ELISA values (E_{405}) in positive clover samples reached 1.752 and 0.484 and in positive controls 0.343 and 2.167 for AMV and ClYVV, respectively. Healthy controls as well as plants inoculated with ClYMV (cvs Start, Beskyd, Nodula – each of 60 plants inoculated, cv. Bivoj – 44 plants, cv. Rezista – 59, cv. Sprint – 58), cytorhabdovirus (cvs Start, Beskyd, Bivoj, Rezista – each of 60 plants inoculated, cv. Sprint – 59), AMV (cv. Sprint – 60), and ClYVV (cv. Fresko – 18) revealed no symptoms as well as no positive reaction by either DAS-ELISA or RT-PCR for determining of possible presence of cytorhabdovirus.

DISCUSSION

The study results in three important conclusions: (*i*) Not all virus species are equally suitable for artificial inoculation of red clover seedlings; WClMV and RCMV are the most suitable viruses out of the six viruses examined. (*ii*) The artificial inoculation method can be used to select resistant plants; the ELISA is more reliable than visual selection based on the absence of virus-like symptoms. (*iii*) There is a variation in susceptibility between cultivars; some cultivars are more resistant and can be used as source of resistance for resistance breeding.

Clover seedlings inoculated with WClMV and RCMV revealed typical symptoms as previously described for red clover plants by Berks (1971) and by Valenta and Marcinka (1971), respectively. Our study showed that red clover plants can be virus-infected without displaying symptoms. The findings are nevertheless in contrast to those of Scott (1982) in the United Kingdom, who had

observed that all 96 red and white clover plants in each of 16 and 14 red and white clover cultivars, respectively, that were artificially infected with WClMV, had become ELISA positive even though some showed no symptoms.

According to Valenta and Marcinka (1971), *P. sativum* is suitable for maintaining RCMV cultures and a good source of virus for purification, while *P. vulgaris* is recommended as assay species, since most of *P. vulgaris* cultivars revealed necrotic lesions (NL) on inoculated leaves suitable for virus detection. *P. vulgaris* cultivars Allen, Bergold, Bolero, Bona, Dita, Luna, Maxi, Prinze, Strike showed a few of local NL after artificial inoculation with RCMV also in our experiments, but only cv. Saxa revealed a lot of local chlorotic lesions (data not shown). In experiments presented here, inoculum from symptomatic leaves of *P. vulgaris* cv. Saxa seems to be also effective for mechanical inoculation of clover seedlings (cvs Bivoj, Nodula, Tempus, Dolina).

RCMV and WClMV rank among the most common and economically important viruses in clover worldwide (Edwardson & Christie 1986; McLaughlin & Boykin 1988; Denny & Guy 2009). At the Victorian AgriBiosciences Centre (Australia), transformation using *Agrobacterium tumefaciens* was undertaken to obtain *T. repens* (white clover) plants resistant to WClMV (Ludlow *et al*. 2009). In view of the fact that mechanical inoculation is a successful method for RCMV and WClMV transmission to various clover cultivars, it is possible to use recurrent phenotypic selection to perform resistance breeding. Repeated screening in the entirety of the red clover cultivars assortment allows selection of cultivars or individual plants that are less susceptible to virus infection. The knowledge of virus-induced symptoms presented here should be useful and highly reliable for WClMV and RCMV detection and breeding selection in the conditions of the European temperate zone and countries with a similar climate.

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