Institute of Soil Science and Plant Cultivation, State Research Institute,
Puławy, Poland

ELIMINATION OF *HOP LATENT VIROID* FROM HOP PLANTS BY COLD TREATMENT AND MERISTEM TIP CULTURE

M. Grudzińska, E. Solarska, A. Czubacka, M. Przybyś and A. Fajbuś

Abstract

Cold treatment and meristem tip culture were used for elimination of *Hop latent viroid* from four cultivars of hop (*Humulus lupulus*). Efficiency of the method was compared for hop cultivars, cold treatments of tested plants and time of meristems excision. HLVd was successfully eliminated from infected plants after one month of cold treatment. Good results were also obtained after cooling tested plants during winter. Excising the meristems as soon as possible after ending of plant cooling was an important factor in HLVd elimination. The effectiveness of viroid elimination depended also on hop cultivar. The use of a very sensitive diagnostic method RT-PCR to confirm complete viroid elimination from infected plants resulted in obtaining a population of HLVd-free hop plants, which remained healthy also after dormancy period.

Key words: *Hop latent viroid*, cold treatment, meristem tip culture, dot-blot hybridisation, RT-PCR

Introduction

Hop latent viroid (HLVd) is widespread in hop (*Humulus lupulus*) cultivated in many regions of the world. In Poland the incidence of HLVd infection in commercial hop gardens ranged between 80 and 90%, depending upon a cultivar (Solarska and Grudzińska 2001 b). Despite the absence of obvious morphological symptoms of infection, HLVd is regarded a serious hops pathogen. Practical significance of HLVd has been reported by English and Czech researchers who have shown that viroid infection decreased total cone yield and alpha-acids content in cones (Bar-

Phytopathol. Pol. 40: 21–30

ISSN 1230-0462

bara et al. 1990, Adams et al. 1991, Patzak et al. 2001). Alpha-acids are the most important compounds of hop resins used by brewing industry as bittering agents in the process of beer production.

To prevent or reduce negative effects caused by the viroid, it is necessary to use viroid-free planting material which can be obtained by elimination of the pathogen from infected plants. Cold treatment (Morton et al. 1993) and thermotherapy (Matoušek et al. 1995), commonly used for elimination of viroids from other hosts, have been unsuccessful for HLVd elimination. These methods initially reduced HLVd concentration in hop plants but the infection usually returned to the original level after some time.

HLVd was very widely spread in hops cultivated in Poland. That is why attempts to obtain viroid-free hops were made at the Institute of Soil Science and Plant Cultivation in Puławy. The negative selection method based on search for viroid-free plants among hop plants grown in commercial hop gardens was unsuccessful. All plants identified as viroid-free on the basis of molecular hybridisation results were then tested by RT-PCR and found infected with HLVd (Solarska and Grudzińska 2001 a).

The results presented in this paper show that HLVd can be effectively eliminated from infected hop plants by meristem tip culture. The study was carried out in two stages: in the first part the comparison of viroid elimination efficiency in dependence on cultivar, cold treatment of tested plants and time of meristem excision was investigated, in the second part the health status of hop plants obtained after tissue culture and then grown in the greenhouse was examined. Plants were tested before and after dormancy period.

Materials and methods

Plant material and *in vitro* culture

The study was carried out on four hops cultivars: 'Magnum', 'Iunga', 'Sybilla' and 'Lubelski'. Shoot tips for excision of meristems were obtained from plants originated from dormant hop sets, which were kept at low temperature for one month and then transferred to the greenhouse, and also from plants grown in the field (hop-garden).

Dormant hop sets were obtained in autumn from commercial propagators. A set consisting of a short underground stem and associated roots is the normal commercial planting material. Sets were stored for a month in trays with sand at 4°C. Next they were potted into soil and transferred to a greenhouse. The total number of potted plants was 350. All plants were tested for the presence of HLVd by dot-blot hybridisation after one month of growth in greenhouse under standard conditions (photoperiod: 16 h, temperature: 23°C). These tests confirmed that all tested plants were infected with HLVd.

Shoot tips were collected for excision of meristems from hop plants grown in greenhouse on two dates: January, i.e. at the beginning of sprouting and April, when donor plants were over 1 m high.

Shoot tips from hop plants grown in the field were collected early in spring, i.e. in the third decade of April. Temperature during the period prior to shoot tip collection is shown in Table 1.

Table 1

Mean temperature during the period prior to collection of shoot tips from hop plants grown in the field

Mean temperature (°C)	January			February			March			April		
	I	II	III	I	II	III	I	II	III	I	II	III
Per decade	–7.7	–0.3	–6.8	4.9	–3.7	–2.2	–3.0	6.4	6.1	–0.1	1.7	0.8
Per month	–4.9			–0.3			3.2			0.8		

The plant material was rinsed with running water for 2 h, sterilised in 6% sodium hypochlorite for 5 min and finally rinsed four times with sterile distilled water. Meristems were cut under aseptic conditions from shoots. The explants were < 0.5 mm in length and contained one or two pairs of primordial leaves. The explants were put onto regeneration medium and kept at 24°C with a 16 h photoperiod. After 14 days the regenerated plants were transferred onto propagation medium. Properly developed stems at least 15 mm in length were then transferred onto rooting medium (Kremheller et al. 1989). All plants were tested for the presence of HLVd by dot-blot hybridisation after two months of *in vitro* growth. The samples, in which viroid was not detected by this method, were checked by RT-PCR. HLVd-free plants were propagated under *in vitro* conditions.

The *in vitro* rooted HLVd-free hop plants were potted in peat and kept in a chamber in high humidity (90%). Next the plants were replanted into soil and moved to greenhouse (photoperiod: 12 h, humidity: 80%, temperature: 21°C). Plants within each cultivar were randomised into groups A or B. Plants in the group A were tested for the presence of HLVd by RT-PCR before and after a dormancy period, plants in the group B were tested by this method only after the dormancy period. Tests before the dormancy period were carried out after two months of plants growth in greenhouse, i.e. eight–nine months after isolation of meristems from tested plants. Plants from A group in which HLVd was detected before the dormancy period were eliminated. Remaining plants from A group and all the plants from group B were cut off and placed outside greenhouse to undergo winter dormancy. After one month of the dormancy period plants were transferred to the greenhouse and after reaching the height of ca 50 cm they were all tested for the presence of the viroid by RT-PCR.

Total nucleic acids (TNA) extraction

TNA were extracted from fresh hop leaves and petioles. The samples from *in vitro* grown plants were tested separately, whereas pooled samples from four plants were taken in the case of plants grown in greenhouse, both before and after the dormancy period. In this case the leaves and petioles were collected from the lowest part of the stem. The amount of reagents used for extraction of TNA from pooled samples are presented in square brackets.

80–100 mg [4 × 200 mg] of plant tissue was ground in 0.65 ml [2.0 ml] of 2% CTAB buffer (100 mM Tris HCl, pH 8.0, 2 M NaCl, 25 mM EDTA, 2% CTAB, 2% 2-mercaptoethanol) (Kiefer et al. 2000) and the homogenate was incubated for 1.5 h at 65°C. Following incubation, 0.85 ml of chloroform/isoamyl alcohol (24:1) was added to the tubes, mixed and centrifuged at 14 000 rpm for 10 min. The aqueous phase was transferred to a fresh tube and an equal volume of chloroform was added. After centrifugation at the same conditions the chloroform extraction was repeated. The total nucleic acids present in the aqueous phase were precipitated with 0.6 volume of cold isopropanol and 0.1 volume of 3 M sodium acetate overnight at 4°C and centrifuged (14 000 rpm, 30 min). The pellet was washed in a 70% solution of cold ethanol and dissolved in ribonuclease free water. The concentration of RNA was estimated by measuring absorbance at 260 nm.

Diagnostic tests

When the result of the dot-blot hybridisation test for viroid presence was positive, plants included in the pooled sample were again tested separately by RT-PCR. Detection of HLVd in the pooled samples was earlier checked on the experimental ones that consisted of a sample derived from HLVd infected plant mixed with samples derived from healthy plants. Hop plants grown *in vitro* were tested by dot-blot hybridisation. TNA extracts (10 μl) were mixed with an equal volume of 37% formaldehyde and 20 × SSC (2:3 v/v). Samples were incubated for 15 min at 60°C and after chilling on ice 2 μl were spotted onto a dry nylon membrane (Hybond-N+, Amersham) and fixed by UV irradiation. Blotted membranes were subjected to molecular hybridisation with HLVd specific DIG-labelled DNA probe (Grudzińska and Solarska 2004). Colorimetric detection was performed at room temperature using anti-digoxigenin-alkaline phosphatase conjugated antibody and NBT/BCIP as the enzyme substrate according to the protocol recommended by the producer (DIG Nucleic Acid Detection Kit, Roche Molecular Biochemicals).

TNA samples in which HLVd was not detected by molecular hybridisation and samples isolated from hop plants grown in greenhouse were amplificated by RT-PCR. HLVd specific primers were used (Matoušek et al. 1995) and RT-PCR was performed according to the protocol described previously (Solarska and Grudzińska 2001 a). The products of the reaction were analysed by electrophoresis through a 3% agarose gel containing ethidium bromide.

Results

In vitro culture of meristems deriving from tested plants cooled under controlled conditions and then transferred to greenhouse and from tested plants grown in the field resulted in HLVd-free hop plants within all the hop cultivars studied (Table 2).

The preliminary results of HLVd elimination from infected hop plants were obtained after testing *in vitro* hop plants by dot-blot hybridisation (Table 2). When RT-PCR was used for examination of the samples, in which the viroid was not detected by molecular hybridisation, additional plants within each cultivar were found to be infected (Table 2).

Table 2

Detection of HLVd by dot-blot hybridisation and RT-PCR in hop plants grown *in vitro* derived from tested plants cooled under artificial and natural conditions

Cultivar	Tested plants	Healthy plants	
		dot-blot hybridisation	RT-PCR
‘Magnum’	146	48	43
‘Iunga’	94	77	71
‘Sybilla’	91	63	60
‘Lubelski’	85	27	25

The efficiency of viroid elimination from infected hop plants by meristem tip culture differed depending on a cultivar, cold treatment of tested plants and time of meristems excision. The method was the most effective for hop plants of cv. ‘Sybilla’ derived from meristems isolated in early spring from plants grown in the field – almost 90% viroid-free plants were obtained after meristem tip culture (Fig. 1). The efficiency of HLVd elimination from plants of the cultivar was also high for plants obtained from tested plants treated with low temperature for one month and grown in greenhouse, but only when meristems were excised from very young plants (in the first term). Collection of meristems from older plants resulted in less than 10% HLVd-free ‘Sybilla’ plants. Among the plants obtained from meristems of plants grown in greenhouse the greatest number of viroid-free plants were observed within ‘Iunga’ cultivar but only after the first date of meristems collection. The efficiency of viroid elimination from plants of the cultivar grown in the field was also very high. No viroid-free plants were found within ‘Lubelski’ and ‘Magnum’ cultivars originating from meristems excised from older plants grown in greenhouse (Fig. 1).

Testing hop plants obtained after *in vitro* culture carried out after two months of their growth in the greenhouse, i.e. eight–nine months after isolation of meristems from tested plants, has shown that only few plants of each cultivar were infected with HLVd (Table 3). After removing infected plants and retesting the rest of the group after the dormancy period, no viroid-infected plants were found (Table 3).

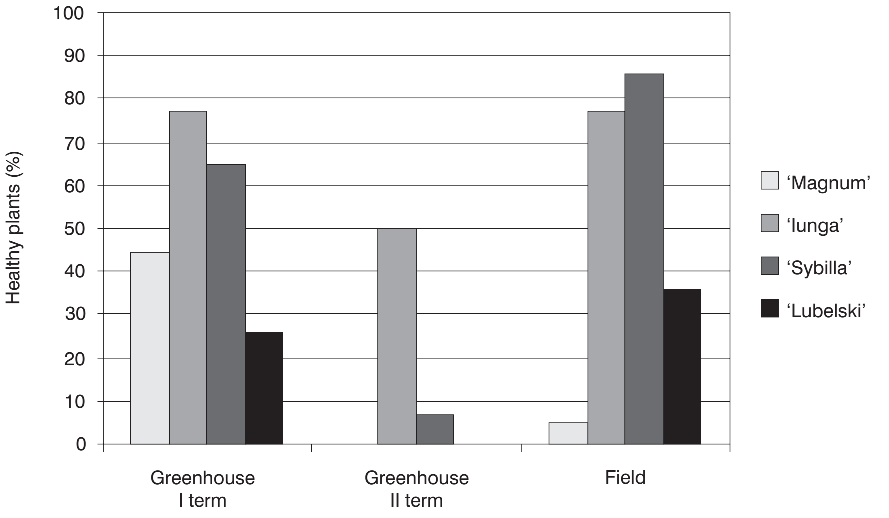

Fig. 1. Efficiency of HLVd elimination from hop cultivars by *in vitro* culture in dependence on treatment of tested plants (plants after one month low temperature therapy grown in the greenhouse and plants from the field after cooling under natural conditions) and time of meristem excision (I term – January, beginning of sprouting, II term – April, plants ca 1 m high)

Table 3

The health status of hop plants obtained after meristem *in vitro* culture and grown in the greenhouse, tested for the presence of HLVd before and after the dormancy period (group A)

Cultivar	Plants tested before dormancy period		Plants tested after dormancy period	
	tested	healthy	tested	healthy
'Magnum'	108	107	107	107
'Iunga'	88	88	88	88
'Sybilla'	95	94	94	94
'Lubelski'	3	2	2	2

Table 4

The health status of hop plants obtained after meristem *in vitro* culture and grown in the greenhouse, tested for the presence of HLVd after the dormancy period (group B)

Cultivar	Tested plants	Healthy plants	
		number	%
'Magnum'	124	117	94.3
'Iunga'	149	144	96.6
'Sybilla'	103	102	99.0
'Lubelski'	256	235	91.8

The percentage of HLVd-free plants in the group of plants tested only after the dormancy period was very high, however, in none of the studied cultivars reached 100% (Table 4).

Discussion

Low-temperature treatment of plants before excising the meristems is one of the methods used for elimination of viroids from diseased plants (Lizarraga et al. 1980, Paduch-Cichal and Kryczyński 1987). The method is recommended for viroids which are resistant to high temperature (PSTVd, CSVd, HLVd). Adams et al. (1996) have tested different periods of low temperature therapy in order to eliminate HLVd from infected hop plants. The highest number of HLVd-free plants was obtained from plants kept at low temperature for eight months, whereas very long cold treatment, up to 21 months, resulted in the lowest recovery of healthy plants. Our study has shown that the cold treatment of plants for one month was sufficient for successful elimination of HLVd from infected plants. Moreover, good results in freeing plants from HLVd were also obtained when the meristem tips were sampled from plants grown in the field in early spring, i.e. in April, which indicates that the cooling of tested plants during winter had an effect similar to that of cold treatment under artificial conditions. It seems that the most important factor for effectiveness of HLVd elimination was a very early growth stage of plants prior to excision of meristems. It is possible that isolation of meristems from the first sprouting shoots resulted in a high percentage of HLVd-free hop plants because the period from the end of plant cooling to meristems excision was too short to allow the pathogen to spread in plants. Hop is a herbaceous perennial and at the end of the growing season all the aerial growth dies back to rootstock – a specialised structure comprised of a short underground stem and associated roots (Neve 1991). When grown commercially, the aboveground part of plant is cut off and the rootstock remains under ground during winter. In HLVd infected plants the viroid survives in the rootstock and in spring spreads slowly into the new shoots, therefore in shoot tips of young plants the level of viroid is very low (Morton et al. 1993). Moreover, Matoušek et al. (1995) has shown that the low level of HLVd in stem apex of young plants was correlated with high activity of a nuclease degrading double-stranded (ds)RNA. During plant growth the concentration of HLVd in shoot tips increases, which causes difficulties in efficient elimination of viroid from older infected hop plants.

The obtained results indicate that the effectiveness of viroid elimination by cold therapy and meristem *in vitro* culture may depend on the hop cultivar. The influence of cultivar on obtaining viroid-free plants by this method has been observed previously. Adams et al. (1996) reported that the percentage of HLVd-free plants obtained after meristem tip culture ranged from 12 to 85, depending on clones and cultivars. They suggested that this variation could also have been caused by differences in treatment of tested plants. In our study the differences in the percentage of healthy plants were observed between similarly treated plants of tested cultivars, which can support suggestion that genetic effect was important for the effectiveness of viroid elimination by meristem *in vitro* culture.

A wide range of HLVd elimination efficiency with respect for different hop cultivars was also achieved by Patzak and Matoušek (1999), however, the percentage of healthy plants was very low (2.4–10.7%). The differences were also influenced by the time of shoot tip sampling. The authors suggested that the optimum time for isolation of meristem tips from plants was time before training, i.e. the end of May and the beginning of June. Our results indicate that the viroid can be more effectively eliminated if meristems are isolated very early in the season, at the beginning of plant sprouting.

Production of viroid-free plants requires the use of sensitive diagnostic methods to detect the viroid presence at a low concentration in plant tissue. A rapid, sensitive and low cost method to test a large number of samples is desirable at the beginning of obtaining healthy material. For this first step of diagnosis the molecular hybridisation is sufficient. To prove complete viroid elimination from infected plants more sensitive diagnostic method, such as RT-PCR, should be used. Similarly, procedure of testing plants obtained by meristem *in vitro* culture have been used by Patzak and Matoušek (1999).

Detection of the few plants infected with HLVd after two months of their growth in greenhouse, i.e. eight–nine months after isolation of meristems from tested plants, indicates that in plants grown *in vitro* viroid was present at a very low concentration, undetectable even by RT-PCR. Under favourable conditions in the greenhouse the viroid have replicated to the detectable level. The increased detectability of HLVd in hop plants after transfer of plants from *in vitro* culture to pots was also reported by Adams et al. (1996).

Our research resulted in HLVd-free hop plants which were grown in greenhouse and remained healthy after the dormancy period. This is in opposition to previous reports which showed that after the dormancy period the number of healthy plants decreased (Morton et al. 1993, Adams et al. 1996). Using RT-PCR instead of molecular hybridisation to prove viroid elimination from infected plants and removing them allowed obtaining a population of healthy hop plants.

After the dormancy period the viroid was also detected but sporadically, only in plants which were not tested before the dormancy period. This points the necessity of frequent and detailed diagnosis of plants grown in greenhouse, especially when they are destined for further propagation.

Patzak and Matoušek (1999), who obtained HLVd-free plants by meristem *in vitro* culture, have reported that hop plants remained viroid-free after two years of *in vitro* cultivation. Our study has shown that HLVd can be efficiently eliminated from infected hop plants by meristem *in vitro* culture and that there is a possibility to maintain a healthy material in greenhouse, not only under *in vitro* conditions. It should be stressed that maintenance of special precautions during the work with healthy plants and their frequent testing are very important for success.

Long-time observation of the health status of hop plants grown in the greenhouse will be continued.

Streszczenie

UWALNIANIE ROŚLIN CHMIELU OD UTAJONEGO WIROIDU CHMIELU METODĄ CHŁODZENIA I KULTUR MERYSTEMÓW WIERZCHOŁKOWYCH

Celem pracy było określenie efektywności uwalniania roślin czterech odmian chmielu od HLVd poprzez chłodzenie i hodowlę merystemów w kulturach *in vitro*. Porównano wydajność metody w zależności od odmiany, sposobu chłodzenia badanych roślin oraz terminu pobierania merystemów wierzchołkowych. Część roślin była poddawana działaniu niskiej temperatury (4°C) przez okres jednego miesiąca, a następnie przetrzymywana w szklarni. Z roślin tych izolowano merystemy w dwóch terminach. Pozostałe rośliny wzrastały na polu i merystemy pobrano z nich wczesną wiosną. Rośliny uzyskane z kultur *in vitro* były badane na obecność wiroidu metodą hybrydyzacji typu dot-blot. Próbki, w których przypadku uzyskano wynik negatywny, ponownie przetestowano za pomocą RT-PCR. Badania wykazały, że skuteczność uwalniania roślin chmielu od HLVd zależy od odmiany chmielu, chłodzenia roślin oraz ich fazy rozwojowej w trakcie pobierania merystemów. Metoda okazała się najbardziej efektywna w odniesieniu do roślin odmiany 'Sybilla' rosnących w polu. W wyniku hodowli pobranych z nich merystemów otrzymano 90% roślin wolnych od wiroidu. Podobnie duży udział roślin zdrowych stwierdzono w przypadku izolacji merystemów w pierwszym terminie z młodych roślin rosnących w szklarni. Niezależnie od sposobu chłodzenia najważniejszym czynnikiem wpływającym na skuteczność uwalniania roślin chmielu od HLVd było pobieranie merystemów w jak najwcześniejszym terminie od zakończenia oddziaływania niskiej temperatury na rośliny. Wykorzystanie chłodzenia w połączeniu z metodą kultur merystemów wierzchołkowych pozwoliło uzyskać dużą liczbę roślin wolnych od HLVd, z których po zastosowaniu bardzo czułej metody diagnostycznej wyselekcjonowano populację całkowicie zdrowych roślin chmielu. Rośliny te pozostały wolne od wiroidu również po okresie spoczynku.

Literature

Adams A.N., Barbara D.J., Morton A., 1991: Effects of hop latent viroid on weight and quality of the cones of the hop cultivar Wye Challenger. Test of agrochemicals and cultivars. 12. Ann. Appl. Biol. 118, Suppl.: 126–127.

Adams A.N., Barbara D.J., Morton A., Darby P., 1996: The experimental transmission of hop latent viroid and its elimination by low treatment and meristem culture. Ann. Appl. Biol. 128: 37–44.

Barbara D.J., Morton A., Adams A.N., Green C.P., 1990: Some effects of hop latent viroid on two cultivars of hop (*Humulus lupulus*) in the UK. Ann. Appl. Biol. 117: 359–366.

Grudzińska M., Solarska E., 2004: Comparison of non-radioactive molecular hybridization methods and RT-PCR for the detection of *Hop latent viroid* on hops. Acta Hortic. 656: 187–191.

Kiefer E., Heller W., Ernst D., 2000: A simple and efficient protocol for isolation of functional RNA from plant tissues rich in secondary metabolites. Plant Mol. Biol. Rep. 18: 33–39.

Kremheller H.Th., Ehrmaier H., Gmelch F., Hesse H., 1989: Production and propagation of virus-free hops in Bavaria, Federal Republic of Germany. In: Proceedings of the International Workshop on

Hop Virus Diseases Rauischholzhausen 1988. Deutsche Phytomedizinische Gesellschaft. Ed. A. Eppler. Ulmer, Stuttgart: 131–134.
Lizarraga R.E., Salazar L.F., Roca W.M., Schilde-Rentschler L., 1980: Elimination of potato spindle tuber viroid by low temperature and meristem culture. Phytopathology 70: 754–755.
Matoušek J., Trněná L., Svoboda P., Oriniaková P., Lichtenstein C.P., 1995: The gradual reduction of viroid levels in hop mericlones following heat therapy: a possible role for a nuclease degrading dsRNA. Biol. Chem. Hoppe-Seyler 376: 715–721.
Morton A., Barbara D.J., Adams A.N., 1993: The distribution of hop latent viroid within plants of *Humulus lupulus* and attempts to obtain viroid-free plants. Ann. Appl. Biol. 123: 47–53.
Neve R.A., 1991: Hops. Chapman and Hall, London.
Paduch-Cichal E., Kryczyński S., 1987: A low temperature therapy and meristem-tip culture for eliminating four viroids from infected plants. J. Phytopathol. 118: 341–346.
Patzak J., Matoušek J., 1999: The elimination of hop latent viroid (HLVd) infection by meristem *in vitro* culture of hop (*Humulus lupulus* L.). In: Proceedings of the Scientific Commission of International Hop Growers' Convention. Ed. E. Seigner. Bayerische Landesanstalt für Bodenkultur und Pflanzenbau, Hüll: 124–129.
Patzak J., Matoušek J., Krofta K., Svoboda P., 2001: Hop latent viroid (HLVd)-caused pathogenesis: effect of HLVd infection on lupulin composition of meristem culture-derived *Humulus lupulus*. Biol. Plant. (Prague) 44, 4: 579–585.
Solarska E., Grudzińska M., 2001 a: Attempts to obtain viroid-free hop plants in Poland. Acta Hortic. 550: 327–330.
Solarska E., Grudzińska M., 2001 b: The field distribution of Hop latent viroid within Polish hop cultivars. Acta Hortic. 550: 331–335.

Authors' address:
Dr. Magdalena Grudzińska, Prof. Dr. hab. Ewa Solarska, Anna Czubacka M.Sc., Marcin Przybyś M.Sc., Agnieszka Fajbuś M.Sc., Institute of Soil Science and Plant Cultivation, State Research Institute, ul. Czartoryskich 8, 24-100 Puławy, Poland, e-mail: mgrudzinska@iung.pulawy.pl

Accepted for publication: 6.06.2006