Journal of Chromatography B, 878 (2010) 1285–1289

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Enantioseparation and plant virucidal bioactivity of new quinazoline derivatives with α-aminophosphonate moiety☆

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ARTICLE INFO

Article history:
Received 29 August 2009
Accepted 12 November 2009
Available online 18 November 2009

Keywords:
Quinazoline
α-Aminophosphonate
Enantioseparation
Bioactivities
Chiralpak IA column
Chiralpak IC column

ABSTRACT

Enantiomers of some new quinazoline derivatives bearing α-aminophosphonate moiety were separated under normal-phase conditions on two immobilized polysaccharide-based chiral stationary phases (Chiralpak IA and Chiralpak IC). The role of two chiral stationary phases (CSPs), polar modifier and column temperature on retention time and separation factor was studied. Apparent thermodynamic parameters were deduced from Van't Hoff plots and plausible mechanism of chiral recognition has been discussed. The semi-preparative separation of some compounds was executed successfully in n-hexane/isopropyl alcohol (IPA) on the Chiralpak IA column. The preliminary bioassay showed that both the enantiomers of the investigated series of compounds possessed similar anti-tobacco mosaic virus (TMV) activities.

1. Introduction

The quinazoline ring system has been found to be an important constituent of many bioactive compounds with potent fungicidal, plant virucidal and antitumor activities [1–4]. As certain derivatives of α-aminophosphonates are also employed as plant growth regulators, fungicides, plant virucides and herbicides [5–10], tactful incorporation of α-aminophosphonate moiety into the parent heterocyclic scaffold of quinazoline might lead to the development of lead structure with high medicinal and/or antiviral activity. Fascinated by this prospect, we synthesized new quinazoline compounds with α-aminophosphonate moiety bearing a chiral center. In majority of the cases, only one of the enantiomers present in the racemate displays bioactivity, while the other may elicit practically negligible or different biological responses with sometimes catastrophic consequences. Therefore, enantiomeric semi-preparative separation of racemic α-aminophosphonates into their pure R or S components using suitable chiral HPLC column for the purpose of biological evaluation has assumed enormous significance. In this context, chiral separations of some α-aminophosphonate derivatives were executed on different Pirkle and derivatized cyclodextrin and polysaccharide-based chiral stationary phases (CSPs) [11–25]. Among these CSPs, Pirkle and polysaccharide-based ones have proved to be more effective for the determination of the enantiomeric purity of aminophosphonic acid derivatives.

Interestingly, polysaccharide-derived CSPs can serve as powerful materials for obtaining enantioseparation in analytical and preparative work due to their broad application and remarkable loading capacity [26–29]. It is also worth noting that Chiralpak IA column and Chiralpak IC column improve chiral separation considerably due to their immobilized characteristics [30–31] coupled with acceptability to wider range of solvents. Although there seems to be no dearth of literature on α-aminophosphonate derivatives, evaluation of their chiral separation on new CSPs still offers a challenging task to an analytical chemist. In this paper, enantioseparation of a series of novel quinazoline derivatives on Chiralpak IA and Chiralpak IC column is presented. Since thermodynamic study is often employed to investigate chiral recognition mechanism [32–37], a non-linear plot of $\ln k$ or $\ln \alpha$ against $1/T$ (Van't Hoff plots) might help to obtain the isoenantioselectivity temperature (T_{iso}) to observe a reversal of elution order of two enantiomers [38–42]. Based on this concept, apparent thermodynamic parameters are also estimated from Van't Hoff plots on Chiralpak IA column. Six pure enantiomers were obtained on semi-preparative Chiralpak IA column, the preliminary bioactivities of enantiomers and their racemates were tested.

☆ This paper is part of the special issue 'Bioanalysis of Organophosphorus Toxicants and Corresponding Antidotes', Harald John and Horst Thiermann (Guest Editors).

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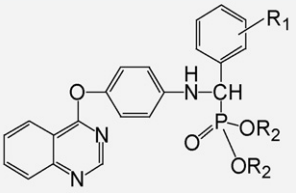
doi:10.1016/j.jchromb.2009.11.024

Table 1
The structures of the studied compounds.

	No.	R_1	R_2
	**1**	*o*-F	CH_2CH_3
	2	H	CH_2CH_3
	3	*o*-F	$CH_2CH_2CH_3$
	4	H	$CH_2CH_2CH_3$
	5	3,4,5-TrisOCH_3	CH_2CH_3
	6	3,4,5-TrisOCH_3	$CH_2CH_2CH_3$

2. Experimental

2.1. Chemicals

Racemic quinazoline derivatives containing an α-aminophosphonate moiety were synthesized in pure form at our own laboratory. The products were unequivocally characterized by NMR spectral data and elemental analysis. The structures are shown in Table 1. The n-hexane, ethanol (EtOH) and 2-propanol (IPA) were of HPLC grade and purchased from Jiangsu Hanbang Sci & Tech. Co. Ltd. (Jiangsu, China).

2.2. Equipment

The analytical HPLC of the compounds was performed on Agilent 1100 series Apparatus composed of a quarternary pump, an autosampler, a DAD detector, a vacuum degasser, a column oven and Agilent Chemstation software. The two columns employed were Chiralpak IA-amylose tris-(3,5-dimethylphenylcarbamate) immobilized on silica-gel and Chiralpak IC-cellullose tris-(3,5-dichlorophenylcarbamate) immobilized on 5 μm silica-gel (both the columns of 250 mm × 4.6 mm i.d., 5 μm, Daicel Chemical Industries Ltd.). Semi-preparative HPLC was carried out by Agilent 1100 series consisting of a preparative pump, a DAD detector and a manual injector with a 10 ml sample loop. Semi-preparative Chiralpak IA column (250 mm × 10 mm i.d., 5 μm) was also purchased from Daicel Chemical Industries Ltd.

2.3. Chromatographic conditions

The analytical samples were dissolved in EtOH at about 0.2–1 mg/ml, injection volume was 5 μl. The mobile phases were composed of n-hexane/IPA (70:30, v/v) and n-hexane/EtOH (70:30, v/v) for two analytical columns. Flow rate was set at 1.0 ml/min and detection wavelength was fixed at 210 nm. Temperature was kept at 25 °C except where effect of temperature was studied. The hold-up time was determined from the elution of an unretained marker (toluene). The enantiomeric elution order on the columns was evaluated by analyzing non-racemic samples enriched by the fraction F1 enantiomer which was obtained on semi-preparative IA column with the mobile phase n-hexane/IPA (60/40, v/v).

The semi-preparative samples were dissolved in mobile phase n-hexane/IPA (60/40, v/v) at 1.5–2.5 mg/ml with an injection volume of 4–8 ml, flow rate 4.0 ml/min and detection wavelength 210 nm. After semi-preparative separation, the collected fractions were analyzed by analytical IA column for determining enantiomeric excess (e.e.).

2.4. Antiviral biological assay

2.4.1. Purification of tobacco mosaic virus

Using Gooding's method [43], the upper leaves of *Nicotiana tabacum* L. inoculated with TMV were selected, ground in phosphate buffer, and then filtered through a double-layer pledget. The filtrate was centrifuged at 10,000 *g*, treated twice with PEG, and centrifuged again. The whole experiment was carried out at 4 °C. Absorbance values were estimated at 260 nm using an ultraviolet spectrophotometer.

$$\text{Virus concentration} = \frac{A_{260} \times \text{dilution ratio}}{E_{1\,\text{cm}}^{0.1\%,\,260\,\text{nm}}} \quad (1)$$

2.4.2. Curative effect of compounds against TMV in vivo

Growing leaves of *N. tabacum* L. of the same ages were selected. The tobacco mosaic virus (concentration of 6×10^{-3} mg/ml) was dipped and inoculated on the whole leaves. Then the leaves were washed with water and dried. The compound solution was smeared

Table 2
k_1, k_2, α and *Rs* values of chiral separation of six compounds on the two columns.

Mobile phase	No.	R_1	R_2	Chiralpak IA				Chiralpak IC			
				k'_1	k'_2	α	*Rs*	k'_1	k'_2	α	*Rs*
n-Hexane–isopropanol (70:30, v/v)	**1**	*o*-F	CH_2CH_3	1.70[a]	2.90[b]	1.71	7.06	3.66[a]	3.80[b]	1.04	0.54
	2	H	CH_2CH_3	2.26[a]	2.93[b]	1.30	3.54	5.07[a]	5.72[b]	1.13	1.81
	3	*o*-F	$CH_2CH_2CH_3$	1.51[a]	3.25[b]	2.15	9.92	2.70[a]	2.97[b]	1.10	1.29
	4	H	$CH_2CH_2CH_3$	2.05[a]	3.23[b]	1.58	6.33	3.84[a]	4.68[b]	1.22	2.88
	5	3,4,5-Trisoch$_3$	CH_2CH_3	1.76[a]	4.28[b]	2.43	10.20	11.69[b]	15.44[a]	1.32	3.59
	6	3,4,5-TrisOCH_3	$CH_2CH_2CH_3$	1.49[a]	3.30[b]	2.21	9.14	8.30[b]	10.67[a]	1.29	3.12
n-Hexane–EtOH (70:30, v/v)	**1**	*o*-F	CH_2CH_3	1.31[a]	1.93[b]	1.47	3.95	1.84[b]	2.18[a]	1.18	2.58
	2	H	CH_2CH_3	1.88	1.88	1	0	2.51[b]	2.72[a]	1.08	1.32
	3	*o*-F	$CH_2CH_2CH_3$	1.10[a]	1.89[b]	1.72	5.45	1.49[b]	1.64[a]	1.10	1.36
	4	H	$CH_2CH_2CH_3$	1.53[a]	1.89[b]	1.23	2.52	2.10	2.10	1	0
	5	3,4,5-TrisOCH_3	CH_2CH_3	1.33[a]	2.48[b]	1.86	6.80	4.08[b]	5.11[a]	1.25	3.63
	6	3,4,5-TrisOCH_3	$CH_2CH_2CH_3$	1.11[a]	2.08[b]	1.87	6.36	3.17[b]	3.76[a]	1.19	2.62

Detection: 210 nm, flow rate: 1.0 ml/min, injection volume: 5 μl, temperature: 25 °C.
The samples were not racemic, enriched by the fraction F1 enantiomer obtained on semi-preparative IA column with n-hexane/IPA (60/40, v/v).
[a] Denotes the first eluted enantiomer on IA column with n-hexane/isopropanol (70:30, v/v) at 25 °C.
[b] Denotes the second eluted enantiomer on IA column with n-hexane/isopropanol (70:30, v/v) at 25 °C.

Table 3
Influence of different temperatures on chiral separation for all compounds.

T (°C)	Compound **1**			Compound **2**			Compound **3**			Compound **4**			Compound **5**			Compound **6**		
	k'_1	α	Rs	k'_1	α	Rs	k'_1	α	Rs	k'_1	α	Rs	k'_1	α	Rs	k'_1	α	Rs
10	2.43	1.88	7.29	3.44	1.35	3.53	2.15	2.38	9.76	3.09	1.67	6.10	2.65	3.28	9.92	2.21	2.83	9.72
20	1.93	1.77	7.15	2.56	1.31	3.54	1.67	2.21	9.80	2.29	1.60	6.18	1.95	2.70	10.19	1.65	2.39	9.43
25	1.70	1.71	7.06	2.26	1.30	3.54	1.51	2.15	9.92	2.05	1.58	6.33	1.76	2.43	10.20	1.49	2.21	9.14
30	1.59	1.67	6.82	2.08	1.28	3.36	1.40	2.08	9.24	1.88	1.55	6.00	1.60	2.31	9.65	1.36	2.11	8.60
40	1.38	1.59	6.23	1.75	1.26	3.20	1.22	1.98	8.68	1.58	1.51	5.80	1.35	2.03	8.75	1.16	1.86	7.41

Detection: 210 nm, flow rate: 1.0 ml/min, injection volume: 5 μl, mobile phase: n-hexane/IPA (70/30, V/V), IA column(4.6 mm × 250 mm).

on the left side and the solvent was smeared on the right side for control. The local lesion numbers were then counted and recorded 3–4 days after inoculation. For each compound, three repetitions were measured. The inhibition rate of the compound was then calculated according to the following formula (av denotes average, and controls were not treated with compound).

$$\text{Inhibition rate}(\%) = \frac{\text{av local lesion no.of control} - \text{av local lesion no.of drug-treated}}{\text{av local lesion no.of control}} \times 100 \quad (2)$$

3. Results and discussion

3.1. Enantioseparation on Chiralpak IA and Chiralpak IC

Chiral separation of title compounds was investigated on IA column and IC column. The separation data, retention factors (k'), separation factors (α) and resolutions (Rs) are indicated in Table 2. The results showed that IA column with IPA as polar modifier exhibited best enantioselectivity for all the six compounds which could be successfully resolved with selectivities of 1.71, 1.30, 2.15, 1.58, 2.43 and 2.21, respectively. Compound **2** could not be resolved on IA column with n-hexane/EtOH (70/30, v/v), and compound **4** could not be resolved on IC column with n-hexane/EtOH (70/30). Compounds **1** and **3** could not be baseline resolved on IC column with n-hexane/IPA (70/30).

On IA column, the separation factors of compounds **1** and **3** (R_1 = F) were obviously better than those of compounds **2** and **4** (R_1 = H) with the same R_2 substituents. It is presumed that the presence of an ortho substituted fluorine atom, due to its strong electron withdrawing ability, can reduce the π electron density of the phenyl ring. On the other hand, electron donating effect of 3,5-dimethyl substituents in IA CSP significantly enhances the π electron density of aromatic ring, which eventually assists in increasing its chiral discriminating capacity. With different R_1 substituents (R_1 either H or F), α does not show any noticeable regular trend on IC CSP. Both the CSPs exhibited better enantioselectivity for compounds **5** and **6**, these compounds with multiple substitutions by methoxy groups, could be more easily discriminated by the chiral column owing to increased π electron density coupled with enhanced steric crowding and favorable hydrogen bond interactions. However, with $R_2 = CH_2CH_2CH_3$ or CH_2CH_3, separation factor α did not reveal any interesting trend on either of these two CSPs.

Chiral recognition capacity of Chiralpak IA was quite high in comparison to Chiralpak IC towards these quinazoline derivatives. Better resolution ability of amylose may be attributed to the helical nature of amylose CSPs possessing well defined chiral grooves as opposed to their cellulose analogues, which presumably appear to be more linear and rigid in nature with shallow grooves [44]. However, the two CSPs exhibited an exceptional level of complementary chiral recognition characteristics for common screening and method optimization strategies [45]. In this experiment, Chiralpak IC showed better chiral resolution capacity for compound **2** with n-hexane/EtOH(70/30) as the mobile phase.

The enantiomeric elution order on the columns was evaluated by analyzing non-racemic samples enriched by the fraction F1 enantiomer which was obtained on semi-preparative IA column with IPA as polar modifier. By comparing the elution orders with IPA as polar modifier on IA column, the elution orders of all compounds were found identical with EtOH as polar modifier on this column. The elution orders of all compounds, however, were completely opposite on IC column with EtOH as the modifier, whereas only compounds **5** and **6** showed this reverse trend on IC column when IPA was used. The results showed that the chiral recognition mechanism is different depending on the nature of CSPs and/or the mobile phases employed.

3.2. Method validation

We decided to validate method on Chiralpak IA column with the mobile phase containing n-hxane/IPA (70/30) at 25 °C. All enantiomers have baseline separation under this condition, as shown in Table 2. Within the concentration range from 0.05 to 1 mg/ml for the racemates, detector response of each enantiomer showed a reasonably well linearity pattern with correlation coefficients between concentration and detector response being >0.99992. The precision of the HPLC method was determined by repeatability (intra-day) and intermediate precision (inter-day) for each racemate with a concentration of 0.5 mg/ml. Method precision had a relative standard deviation (RSD%) (including retention time and peak area) below 2.0% for repeatability and for the intermediate precision.

3.3. Effect of temperature

In order to further optimize the separation condition and study the chiral recognition mechanism, the effects of the column temperature on the retention and enantioseparation were investigated on IA column with IPA as polar modifier. The effect of temperatures was studied in the range 10–40 °C. The results are shown in Table 3. With decreasing temperature, both retention factor (k') and separation factor (α) increased. We could see that the resolution of compounds **2**–**5** attained nearly the highest values around a temperature of 25 °C.

In chromatographic enantioseparation, the relationships between chromatographic data and the column temperature are as follows:

$$\ln k' = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \Phi = -\frac{\Delta H^\circ}{RT} + \Delta S^* \quad (3)$$

$$\ln \alpha = -\frac{\Delta\Delta H^\circ}{RT} + \frac{\Delta\Delta S^\circ}{R} \quad (4)$$

In the above equations (k') is the retention factor, α is the separation factor, R is the gas constant and Φ is the column phase ratio. ΔH and ΔS represent the differences in the enthalpy and entropy respectively when one enantiomer is transferred from mobile phase to the stationary phase. $\Delta\Delta H$ and $\Delta\Delta S$ represent

Table 4
The thermodynamic parameters for the enantioseparation of compounds **1**–**6**.

Compound	**1**	**2**	**3**	**4**	**5**	**6**
$\Delta\Delta H$ (J mol^{-1})	−4140	−1700	−4530	−2470	−11,810	−10,230
$\Delta\Delta S$ (J mol^{-1} K^{-1})	−9.40	−3.54	−8.83	−4.49	−32.00	−27.59
r^2	0.9978	0.9878	0.9963	0.9931	0.9874	0.9935

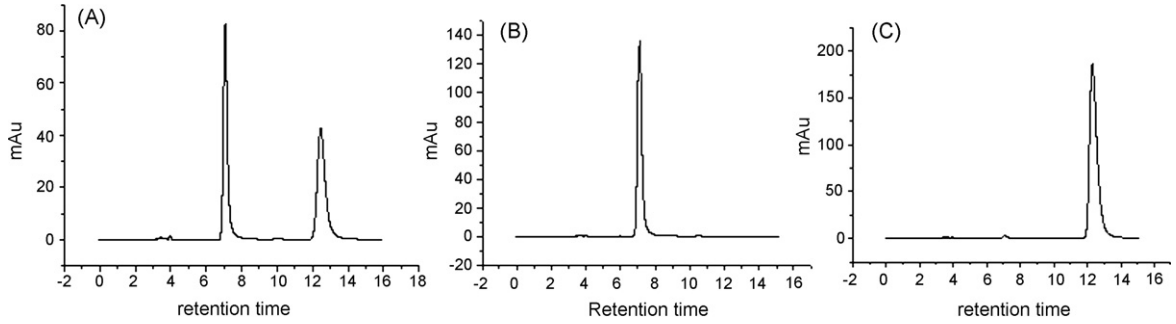


Fig. 1. (A) Analytical chromatogram of compound **5**. (B) Purity determination of the single fraction F1 of compound **5** collected on a semi-preparative scale. (C) Purity determination of the single fraction F2 of compound **5** collected on a semi-preparative scale. Column: IA column (250 mm × 4.6 mm id, 5 μm); injection volume: 5 μl; detection: 210 nm; flow rate: 1.0 ml/min; mobile phase: n-hexane/IPA (60:40, v/v).

Table 5
Enantiomeric excess values and optical rotation values of the first (F1) and the second (F2) fractions collected after semi-preparation.

Compounds	First fractions (F1)		Second fractions (F2)	
	e.e.%	$[\alpha]_D^{20}$	e.e.%	$[\alpha]_D^{20}$
1	100	+28.2	100	−30.3
5	100	+32.6	98	−33.4
6	100	+45.8	99	−43.3

Determination of enantiomeric excess—column: IA column (250 mm × 4.6 mm i.d., 5 μm); injection volume: 5 μl; detection: 210 nm; flow rate: 1.0 ml/min; mobile phase: n-hexane/IPA (60/40, v/v).
Optical rotation measurement: temperature, 20 °C; solvent, methanol.

the differences of ΔH and ΔS respectively for a given pair of enantiomers.

On IA column, the plots of $\ln\alpha$ and $\ln(k')$, against $1/T$ were highly linear in the temperature range of 10–40 °C, which indicated that in this temperature range chiral recognition mechanism for two enantiomers may not change and also the elution order of two enantiomers remained unaffected. It was confirmed by analyzing non-racemic samples enriched by the fraction F1 enantiomer obtained on semi-preparative IA column with n-hexane/IPA (60:40, v/v). The thermodynamic parameters for the enantioseparation of compounds **1**–**6** are shown in Table 4. We could see that $\Delta\Delta H$ and $\Delta\Delta S$ values for all compounds were negative, suggesting that the enantioseparation was enthalpy-driven, and the separation factor decreases with increase in the temperature. As the absolute value of $\Delta\Delta H$ is >1.0 kJ mol^{-1}, we believe that the extra strong π–π type or hydrogen bond interaction between the second-eluting substance and CSP is vital for successful enantioseparation [46]. Furthermore, in accordance with the results described in Section 3.4, it appears that the enhanced π–π type interaction is crucial to chiral discrimination for these compounds on IA column with n-hexane/IPA.

3.4. Effect of the content of IPA

Considering that the enantioseparations of aforementioned six compounds were satisfying with n-hexane/IPA solvent on IA column, we turned our attention to establish the role of IPA content towards retention and enantioseparation. We could notice in the range 60/40 to 80/20 of n-hexane/IPA as the mobile phase, separation factors (α) of all compounds were almost stable on enhancing the solvent polarity. On the other hand, resolution (Rs) and retentions of all compounds decreased with increase in the solvent polarity. The effect of the content of IPA on the enantiosepara-

Table 6
Curative effect of the new compounds against TMV in vivo.[a].

Compound (500 μg/ml)	Inhibition rate (%)		
	Racemates	F1 fraction (+)	F2 fraction (−)
1	53.75* ± 3.8	50.77* ± 4.9	54.29* ± 4.4
2	30.3* ± 5.3	/	/
3	22.7* ± 4.7	/	/
4	29.5** ± 2.0	/	/
5	51.22* ± 2.2	47.39* ± 4.1	46.13* ± 3.7
6	45.85* ± 3.6	46.99* ± 4.5	48.02* ± 3.4
Ningnamycin	55.18* ± 2.9		

[a] All results were expressed as mean ± SD; $n = 3$ for all groups.
* $P < 0.05$.
** $P < 0.01$.

tion was not significant due to stable separation factors, suggesting that for these compounds hydrogen bond interaction might not be crucial for chiral recognition on IA column with n-hexane/IPA.

3.5. Semi-preparative separation of some enantiomers

Three racemates (compounds **1**, **5** and **6**) of the investigated series of compounds were shown to be antiviral active in the bioassay. In order to determine whether their isolated pure enantiomers reveal differential behavior in terms of antiviral activity, we carried out semi-preparative separation of the three racemates according to the chromatographic conditions described in Section 2.3. Two fractions were collected: the first-eluting enantiomer was collected in F1 fraction and the second-eluting enantiomer was collected in F2 fraction. Fig. 1 shows the chromatograms for purity determination of compound **5**. The analytical assessment of enantiomeric excess values of all collected fractions showed that e.e. was more than 98%. Optical rotation values were measured on a WZZ-ZS automatic polarimeter, the data were listed in Table 5.

3.6. Antiviral activity

The results of in vivo bioassay of active compounds **1**, **5** and **6** against TMV are given in Table 6. Commercially available plant virucide Ningnanmycin, perhaps the most successful registered plant antiviral agent in China, was used as a reference antiviral agent for our study. Compounds **2**–**4** which showed relatively lower curative activities (30.3%, 22.7%, 29.5%, respectively) against TMV at 500 mg/l, were not subjected to enantioseparation. The data provided in Table 6 indicate that the two enantiomers and the corresponding racemates did not exhibit significant difference on anti-tobacco mosaic virus (TMV) activities.

4. Conclusion

New immobilized-type Chiralpak IC CSP and Chiralpak IA CSP were compared for their recognition ability towards six quinazoline derivatives containing an α-aminophosphonate moiety. Chiralpak IA column revealed high enantioselectivity for all compounds with IPA as organic modifier. The effect of the column temperature (10–40 °C) on the enantioseparation indicated that the chiral separation of all compounds was enthalpy-driven, and the separation factors decreased with rise in temperature. Some racemates were subjected to semi-preparative separation with n-hexane/IPA (60/40, v/v) mixture at about 25 °C on semi-preparative IA column. The analytical assessment of the enantiomeric excess values of all collected fractions was higher than 98%. The preliminary bioassay showed that the two enantiomers showed good inhibitory activities and did not exhibit significant difference in anti-tobacco mosaic virus (TMV) activities.

Acknowledgement

The authors wish to thank the National Natural Science Foundation of China (No. 20872021) for the financial support.

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