Research Article

Effects of *mti-2* Transgenic Potato Plants on the Aphid *Myzus persicae* (Sternorrhyncha: Aphididae)

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Overexpressed in transgenic plants, protease inhibitors showed insecticidal effects against several insect taxa. We transformed potato internodes with the mustard trypsin inhibitor *mti-2* gene. Among the 35 independent transgenic potato lines obtained via *Agrobacterium tumefasciens* transformation, four (DM6, DM7, DM11, and DM19) were selected for their high level of MTI-2 (at least to 30% of trypsin activity inhibition). Feeding assays were carried out to evaluate their effects on the green-peach aphid, *Myzus persicae* (Sternorrhyncha: Aphididae). Prereproductive period, nymphal mortality, adult fecundity, and doubling time of *M. persicae* populations were monitored on nontransformed potato plants (NT) and the four selected DM lines. Compared to NT plants, DM19 did not induce any effect on *M. persicae*. In contrast, DM7 and DM11 increased nymphal survival by approximately 20%. DM6 and DM11 lines slightly enhanced *M. persicae* daily fecundity and intrinsic rate of natural increase, leading to a reduction of the doubling time of the populations by 1 day. DM6 did not impact nymphal mortality, whereas with the DM11 almost all the nymphs survived. Potato plants transformed with the mti-2 gene variably affected the life history of *M. persicae* but did not show any insecticidal effect on the aphid.

1. Introduction

Plant protease inhibitors (PIs) have been found in *Brassicaceae*, *Gramineae*, *Fabaceae,* and *Solanaceae* and are constitutively produced in storage organs [1] to mobilize protein reserves [2], and in seeds to regulate endogenous proteinases during dormancy [3–5]. PIs may also be synthesized in plants attacked by insects or pathogens [6–8]. Among the different classes of PIs described in plants, serine PIs are the most studied, the two best-characterized families belonging to the Kunitz-type and Bowman-Birk inhibitor's family (BBI) [9]. Several serine PIs from different plant origin (e.g., cowpea (*Vigna unguiculata*), soybean (*Glycine max*), tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*) and barley (*Hordeum vulgare*) were isolated and tested against insects. In vitro studies showed that chymotrypsin and

trypsin inhibitors from *Nicotiana alata* inhibit gut protease activities of the native budworm *Helicoverpa punctigera* (Lepidoptera) and the black field cricket *Teleogryllus commodus* (Orthoptera), and reduce their movement and growth [10]. The cowpea trypsin inhibitor (CpTI) delayed larval growth and development in *Lacanobia oleracea* (Lepidoptera) [11].

Because of their deleterious effects via in vitro delivery, and owing to possibilities offered by transgenesis, several serine PIs were overexpressed in plant tissues to evaluate their effects on various pests *via* direct expression. Tobacco (*Nicotiana tabacum*), potato, rice (*Oryza sativa*), cotton (*Gossypium* sp.), and cereals were genetically modified with serine (trypsin or chymotrypsin) PIs and the transformed plants were reported toxic against lepidopteran [10–13] and coleopteran pests [14, 15]. CpTI conferred resistance against many lepidopteran (*Heliothis* and *Spodoptera*), coleopteran

(*Diabrotica* and *Anthonomus*), and orthopteran (*Locusta*) pests and induced toxic effects in the Lepidoptera *Lacanobia oleracea* [11]. Tobacco and potato plants expressing a soybean serine PIs caused the death of *Spodoptera littoralis* larvae [16]. Several studies also described the transformation of *Arabidopsis,* tobacco, and oilseed rape plants with the mustard trypsin inhibitor (*mti-2*) encoding gene. Because this inhibitor possesses a specific structure that differs from all the other known serine PIs, it has been considered as a new family [17]. MTI-2 expressing plants were only tested on Lepidoptera and *mti-2* levels expressed in these transgenic plants induced variable resistance against larvae of *Mamestra brassicae, Spodoptera littoralis,* and *Plutella xylostella* reared on transformed *Arabidopsis* and oilseed rape [18–20].

Although the effects of serine PIs are well described in Lepidoptera and Coleoptera, they remain poorly documented in Homoptera. However, it is known that variable effects of serine PIs on aphids depend on the inhibitor, its delivery mode (in vitro or *in planta*), and the aphid species targeted. Trypsin inhibitor genes expressed in rice conferred resistance against the planthopper *Nilaparvata lugens* [21]. Pea (*Pisum sativum*) and soybean trypsin-chymotrypsin inhibitors (respectively PsTI and SbBBI) from the BBI family were also reported to depress growth and survival when delivered in vitro to the aphid *Acyrthosiphon pisum*, whereas CpTI from cowpea had no effect [22]. Nymphal survival of the aphids *Myzus persicae* [23], *Aphis gossypii* [23], and *Macrosiphum euphorbiae* [24] was unaffected by PsTI and SbBBI. The latter enhanced fecundity of *M. euphorbiae* when delivered via an artificial diet at $20\,\mu g/mL$, and it decreased fecundity at $500\,\mu g/mL$ [24]. CHY8, an MTI-2 variant, was shown as the most aphicidal PI within the BBI family. However, it presented a variable inhibitory activity, the lethal concentration of CHY8 being higher for *M. persicae* and *A. gossypii* than for *A. pisum* [23].

M. persicae can transmit more than 100 viruses on plants, including potatoes, one of the most economically important crops in Northern Europe. Diseases caused by these viruses (e.g., PVY, PLRV, PMTV) incur serious yield losses. No antiviral treatment is available to reduce damage and the primary means to control virus spreading are the management of aphid populations. Owing to the potential of MTI-2 expressed in transgenic plants to manage pests, our objective was to evaluate the effects of *mti-2* transgenic potato plants on *M. persicae*, a key pest of several crops.

2. Materials and Methods

2.1. Plant Material and Transformation.
Genetically modified potato plants of *S. tuberosum* cultivar Désirée were obtained by transformation with the *Agrobacterium tumefasciens* strain GV3101 containing the plasmid pKY-MTI-2 [18]. The vector was built with the mustard trypsin inhibitor (*mti-2*) gene associated with the selection gene coding for kanamycin resistance (*nptII*), under the control of the 35S CaMV promoter and the pea Rubisco terminator [18]. Plant transformation, selection and regeneration were carried out as described in Saguez et al. [25]. Briefly, potato

internode explants were cocultivated for 3 days with *A. tumefasciens*. Four weeks later, calli were transferred onto a budding medium supplemented with kanamycin to select transformed lines. After bud regeneration, transgenic lines and a nontransformed line (NT) of the cultivar Désirée were micropropagated and acclimatized in a greenhouse at $20 \pm 1°C$ and under a 16-hour light: 8-hour dark photoperiod. One month after acclimatization, plants were used for feeding assays with *M. persicae*.

2.2. Plant Characterization

2.2.1. Genomic Characterization.
Transgene identification was determined by polymerase chain reaction (PCR). PCR amplifications were realized using two *mti-2* primers (forward: 5′-ATG-GCC-ATG-GCA-AAA-AAA-TC-3′ and reverse: 5′-AAT-GCC-AAC-TCT-TAG-AAT-CT-3′) and two *nptII* primers (forward: 5′-ATC-GGG-AGG-GGC-GAT-ACC-GTA-3′ and reverse: 5′-GAG-GCT-ATT-CGG-CTA-TGA-CTG-3′) as described in Saguez et al. [25]. After PCR, the samples were analyzed by agarose gel electrophoresis to verify amplification.

2.2.2. MTI-2 and Enzymatic Activity.
Protein extracts were prepared with the third and fourth fully expanded leaves from the apex. Transgenic and nontransformed lines were frozen in liquid nitrogen, finely ground and homogenized in a phosphate buffer 0.1 M (NaH_2PO_4/Na_2HPO_4 pH 6). After 15 minutes of 15 000 g centrifugation at $4°C$, the supernatant was collected for further tests.

Inhibitory activity due to MTI-2 expressed in plants was quantified by assaying azocazein hydrolysis, as described in De Leo et al. [18] with slight modifications. Briefly, $5\,\mu g$ of crude protein extract of nontransformed and *mti-2* expressing potato lines were incubated with trypsin solution (2.5 mg/mL) in the presence of azocasein (0.5%) in 50 mM Tris-HCl buffer (pH 8) containing 10 mM of $CaCl_2$. After 2 hours of incubation at $37°C$, azocasein hydrolysis was stopped with 20% trichloroacetic acid (TCA). Following 15 minutes of incubation at $4°C$, proteins were precipitated by centrifugation. The supernatant was collected and enhanced with 1 M NaOH. Optical density was measured at 450 nm.

Active protease inhibitor detection was monitored by zymography technique, incorporating protease substrate into the gel mixture. The protease inhibitors were detected by the method of Uriel and Berges [26], as modified by Garcia-Carreno et al. [27]. Briefly, crude protein extracts were dissolved in nonreducing Laemmli sample buffer and separated by electrophoresis using continuous 12% polyacrylamide gel containing 0.1% of porcine gelatin. After a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the gel was washed twice with 50 mM Tris-HCl buffer (pH 8) containing 2.5% TritonX-100 (v/v) for 15 minutes to remove SDS, followed by a brief incubation in the same buffer without TritonX-100. The gel was then incubated for 1 hour at $37°C$ in Tris buffer (Tris 50 mM pH 8, $CaCl_2$ 5 mM, NaCl 100 mM) containing 0.1 mg/mL of

trypsin. The gel was then stained with Coomassie brilliant blue which allowed the detection of trypsin inhibitors.

Detection of MTI-2 protein in transgenic lines was performed by Western analysis. Five μg of total protein were separated by electrophoresis on a 12% polyacrylamide gel. The proteins were then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Bioscience, Saclay, France). The membrane was soaked 30 minutes in saturation solution of 50 mM Tris pH 8, containing 100 mM NaCl and 0.5% of BSA. The membrane was hybridized with polyclonal anti-MTI-2 antibodies (1/1000), obtained from rabbit and given by Dr. Jouanin, then washed and incubated with secondary antirabbit antibody (1/25000). Presence of the MTI-2 protein was detected by chemiluminescence on an autoradiography film (Hyperfilm ECL, Amersham Bioscience, Saclay, France).

2.2.3. Ionic Characterization.

One month after acclimatization, ion extraction was carried out with sulphuric acid. Ion concentrations in plants were determined by atomic absorption spectrophotometry (Instrumentation Laboratories aa-ae spectrophotometer 257) for Mg^{2+} and Ca^{2+} and flame spectrophotometry (Corning 400) for Na^+ and K^+ assays. Three independent assays were conducted on lyophilized plants.

2.3. Insect and Feeding Bioassays.

The *M. persicae* colony, originated from a single apterous female collected in 1999 in a potato field near Loos-en-Gohelle, France ($50°27'27''$N and $2°47'30''$E), was maintained on leaves of nontransformed potato plants (*S. tuberosum* cultivar Désirée) in a ventilated Plexiglass chamber under parthenogenesis-inducing conditions as described in Saguez et al. [25].

In planta experiments where done with the NT and the four DM lines in which recombinant MTI-2 led to at least 30% trypsin activity inhibition compared to the NT plants. Six plants of each selected DM or NT line were used for insect feeding bioassays. Adults taken from the rearing were placed on an artificial diet during 72 hours to synchronize the offspring [25]. Five young nymphs (<24 hours) were transferred in a microcage ($\varnothing$ 16 × 8 mm) clipped on the abaxial face of a third or fourth fully expanded leaf from the apex. At least 12 replicates were carried out for each line.

For each test, nymphal survival, prereproductive period (i.e., the period of time from birth until onset of reproduction), adult emergence, and offspring were recorded daily. These demographic parameters were used to calculate the daily fecundity, the intrinsic rate of natural increase (r_m), and the doubling time of *M. persicae* populations. The r_m value was calculated according to the Lotka equation and its variance using the "Petitr" software and the doubling time was calculated thanks to the equation: DT = ln 2/r_m, as described by Saguez et al. [25].

2.4. Statistical Analysis.

The effect of MTI-2 transgenic potato lines on nymphal survival was analyzed with the Pearson's χ^2 test. Oneway analysis of variance (ANOVA) was carried out with Statistica 5.5 software (StatSoft, Tulsa,

(a) Gelatine/SDS-PAGE

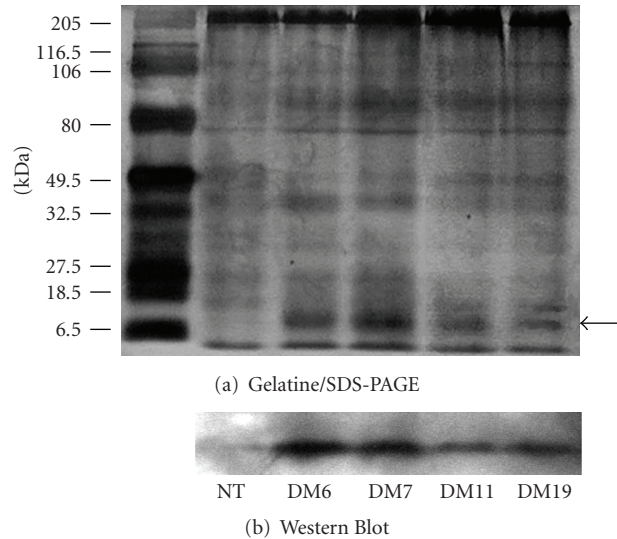NT   DM6   DM7   DM11   DM19

(b) Western Blot

FIGURE 1: Detection of (a) Trypsin inhibitors by gelatin/SDS-PAGE contained in nontransformed (NT) and selected DM lines. Trypsin inhibition by PIs contained in plant extracts was visualized by stains. Arrow indicates MTI-2; (b) MTI-2 by ECL Western blotting in nontransformed (NT) and selected DM lines.

Oklahoma, USA) to test the effects of *mti-2* expression in potato plants on aphid demographic parameters (i.e., prereproductive period, daily fecundity, r_m and population's doubling time). Significant effects were further analyzed with the positive least significant difference test (PLSD) of Fisher at P = .05. Significant variations of Ca^{2+} rates in plants were statistically analyzed by a Newman-Keuls test at P = .05.

3. Results

3.1. Plant Characterization.

Genetic transformation yielded 35 independent transgenic lines, hereafter denoted DM1 to DM35, DM standing for Désirée expressing MTI-2. Electrophoresis of PCR products of the transformed potato lines showed a unique band of approximately 700 bp, confirming that all transgenic lines have been successfully transformed with the *npt-II* gene. Another band of 500 bp revealed the presence of *mti-2* gene insertion in the transformed potato lines. As expected, no *mti-2* fragment was observed in the NT line used as control (results not shown).

Total protein content was statistically equivalent across all potato lines (data not shown). The range of trypsin activity in the presence of leaf protein crude extract varied from 48.6 ± 4.1 to 100.2 ± 1.6 pmol hydrolyzed azocasein/min/mg of trypsin for the 35 DM lines, compared to 93.0 ± 0.2 pmol hydrolyzed azocasein/min/mg of trypsin for the NT line. The percentage of trypsin activity inhibition due to MTI-2 was evaluated by the ratio of trypsin activity in the presence of crude extract from DM lines and NT (data not shown). For insect bioassays, we selected four lines (i.e., DM6, DM7, DM11, and DM19) whereby the protein crude extract exhibited at least 30% of trypsin activity

Table 1: Average (± s.d.) trypsin activity in presence of leaf protein crude extracts from nontransformed (NT) and *mti-2*-expressing potato lines.

Line	Average trypsin activity (pmol hydrolysed azocaseine/min/mg of trypsin)
NT (= 100% activity)	93.0 ± 0.2
DM1	83.7 ± 0.0
DM2	75.1 ± 10.2
DM3	79.0 ± 1.8
DM4	79.5 ± 6.7
DM5	69.8 ± 5.5
DM6*	60.2 ± 10.3
DM7*	48.6 ± 4.1
DM8	81.8 ± 1.4
DM9	66.6 ± 0.3
DM11*	63.2 ± 0.1
DM12	100.2 ± 1.6
DM13	77.9 ± 4.5
DM14	73.8 ± 3.7
DM15	74.4 ± 4.8
DM16	75.0 ± 9.9
DM17	73.1 ± 3.2
DM18	82.1 ± 3.9
DM19*	60.7 ± 9.4
DM20	66.9 ± 3.2
DM21	80.2 ± 3.5
DM22	75.8 ± 0.8
DM23	78.8 ± 8.1
DM24	80.3 ± 11.4
DM26	71.7 ± 2.9
DM28	80.3 ± 4.0
DM29	68.6 ± 0.1
DM30	73.0 ± 5.3
DM31	85.1 ± 11.3
DM32	76.4 ± 13.2
DM33	83.1 ± 5.7
DM34	67.7 ± 7.0
DM35	83.2 ± 0.1

*Selected lines in which at least 30% of trypsin activity were inhibited compared to nontransformed line. Trypsin activity inhibition could not be determined for DM10, DM25, and DM27 lines.

inhibition, compared to the NT line (Table 1). Gelatin/SDS-PAGE revealed several trypsin inhibitors (Figure 1(a)). A major band of approximately 7 kDa was detected in the four selected DM lines while absent in the NT line. This unique band, which corresponded to MTI-2 molecular weight, was also revealed by Western blot analysis only in the DM lines (Figure 1(b)).

No phenological variation was observed between the NT and the four DM lines. Measurement of ion concentrations did not show significant variation of Mg^{2+}, Na^+, and K^+
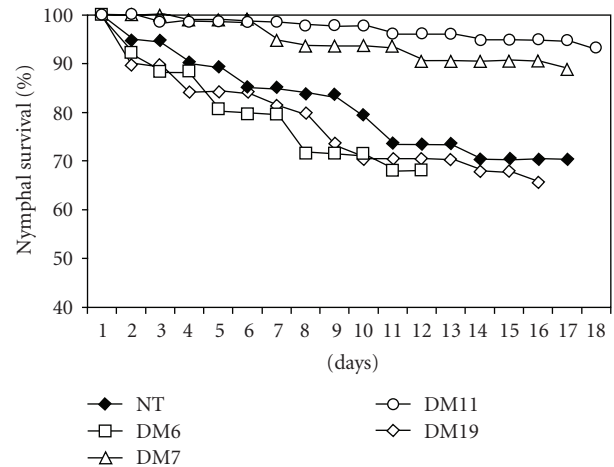

Figure 2: Nymphal survival (%) of *Myzus persicae* reared on nontransformed (NT) and selected DM lines.

concentrations between the NT and the four DM lines. Compared to the NT line, Ca^{2+} concentration increased in DM7 and DM19 (7.51 versus 12.31 and 10.89 mg Ca^{2+}/g dry material, resp.).

3.2. Feeding Bioassays. The four *mti-2* transgenic potato lines induced variable effects on the demographic parameters of *M. persicae*, compared to the NT line. There was no significant change on *M. persicae* nymphal survival on DM6, DM19 (χ^2 = 14.7, df = 11, P <.20; χ^2 = 16.7, df = 15, P < .34, resp.) compared to the NT line. In contrast, nymphal survival significantly increased of approximately 20% which was observed when aphids were reared on DM7 and DM11 (χ^2 = 65.3, df = 16, P <.00; χ^2 = 99.3, df = 16, P < .00, resp.), compared to the NT potato line (Figure 2).

No effect was observed on the mean duration of aphid prereproductive period for all the lines (Table 2). The aphids became adults in an average period of 10 days in both NT and DM lines. Daily fecundity of aphids reared on the foliage of DM7 and DM19 lines was statistically similar to those of aphids fed with the NT line. Subsequently, no variation of r_m and doubling time was observed when these two transgenic potato lines were used. Conversely, DM6 and DM11 led to increased daily fecundity and r_m value when compared to the NT line. As a consequence, the doubling time was significantly reduced by approximately 1 day when aphids were reared on DM6 and DM11.

4. Discussion

Feeding bioassays showed variability in the effects of *mti-2* transgenic potato lines on *M. persicae*, ranging from non-significant (DM19) to probiotic effects (DM6 and DM11). DM19 did not affect the demographic parameters of *M. persicae* compared to the NT line. In contrast, DM6 and DM11, that had a similar range of trypsin activity as DM19, induced slightly but significant probiotic effects on the fitness of adults, increased their daily fecundity, and

TABLE 2: Average (± s.d.) demographic parameters of *Myzus persicae* reared on nontransformed (NT) or selected DM lines.

Demographic parameters	Nontransformed	*mti-2*-expressing potato lines				F^{**}	P^{***}
	Line NT	DM6	DM7	DM11	DM19		
	$n^* = 24$	$n = 31$	$n = 33$	$n = 34$	$n = 16$		
Prereproductive period (days)	$10.3 \pm 1.8^{a\ ****}$	9.7 ± 1.8^a	10.0 ± 1.5^a	9.9 ± 1.7^a	10.8 ± 2.3^a	1.19	<.31
Daily fecundity (nymphs/female/day)	1.2 ± 0.3^a	1.4 ± 0.4^b	1.2 ± 0.3^a	1.4 ± 0.5^b	1.1 ± 0.2^a	3.49	<.01
r_m (females/female/day)	0.14 ± 0.05^a	0.17 ± 0.03^b	0.15 ± 0.04^a	0.17 ± 0.04^b	0.15 ± 0.03^a	2.91	<.02
Doubling time (days)	5.2 ± 1.4^a	4.1 ± 0.9^b	4.7 ± 1.1^a	4.4 ± 1.2^b	5.0 ± 1.5^a	3.41	<.01

*n: number of aphids tested. **F: Fisher's test value of one-way ANOVA analysis. ***P: P value of the Fisher's test. ****Values in the same row followed by the same letter indicate no significant difference according to the PLSD test of Fisher ($P = .05$).

consequently reduced their doubling time. DM7, which exerted the highest inhibition on trypsin activity, increased only nymphal survival of aphids, compared to the NT line. Earlier studies had reported highly variable effects in response to serine PIs ingestion by aphids via in vitro and *in planta* delivery systems [22–24].

The involvement of serine proteases in the digestive processes has been described in Coleoptera [28], Diptera, [29] and Lepidoptera [12] and these gut proteases may be potential targets of serine PIs. However, serine PIs-based strategies to manage aphid populations often failed because aphids have been reported to mainly produce cysteine proteases [30–33]. In view of published studies on serine PIs effects in aphids, our study supports the concept that aphids can be considered as nondigestive targets in the context of an MTI-2-based management strategy.

Aphids feed on phloem sap that contains low concentrations of proteins. Although the *mti-2* gene was expressed under the control of a constitutive promotor, we hypothesize that MTI-2 concentration is lower in the sieve tubes than in the leaves. It has been reported that PIs must constitute at least 1% of total soluble proteins to exert deleterious effects on insects [34, 35]. Consequently, in contrast to coleopteran and lepidopteran pests that feed on all leaf tissues and ingest high amounts of PIs, aphids only ingest phloem sap and may be less exposed to recombinant PIs. This could also explain the lack of or minimal effects in DM7 and DM19, despite the high inhibitory activity of MTI-2 in these lines.

Several proteins [36, 37], including PIs [22, 24, 32], have been reported to cross the digestive epithelial barrier of aphids. The probiotic effects of DM6 and DM11 lines could be due to the crossing of MTI-2 through mesenteric cells from aphid's gut to the hemolymph. In the hemolymph, PIs can interact with different developmental processes, such as growth or reproduction, by amino acid food supplements or by MTI-2 activity in the hemolymph.

Localization of transgene insertion in the plant genome carried out by *A. tumefasciens* is not controlled. Even if no phenological variation was observed between DM and NT lines, gene insertions in the potato genome may have interfered with potato metabolism by inducing pleiotropic effects and modifications of plant physiology that favour the aphids. DM7 and DM19 lines exhibited the highest concentrations of Ca^{2+} and exerted the least effect on aphid demographic parameters when compared to the NT line. The rise of Ca^{2+} rate is reported to be one of the most important

factors in plant defense response. Callose deposition and phloem sap proteins precipitation that occlude the sieve tubes and prevent sap loss in response to aphid attack are Ca^{2+}-dependent [38]. Despite the damage caused to cell membranes, aphids are able to overcome plant defenses by reducing calcium influx, preventing callose formation and protein precipitation by chelating Ca^{2+} [39]. Higher Ca^{2+} concentrations in DM7 and DM19 may have interfered with aphid food intake, confounding the probiotic effects observed on DM6 and DM11 lines.

Our results showed that MTI-2 transgenic potato plants induced no or slightly positive effects on *M. persicae* life history and question the efficacy of transgenic serine PIs to manage aphids.
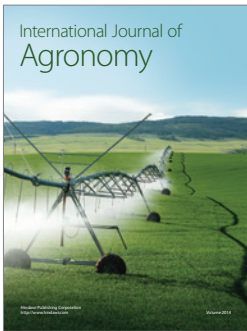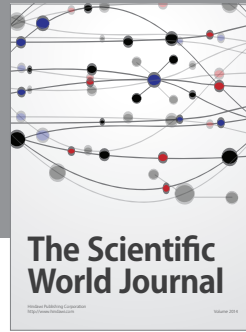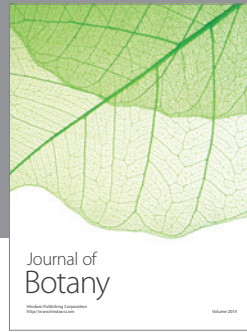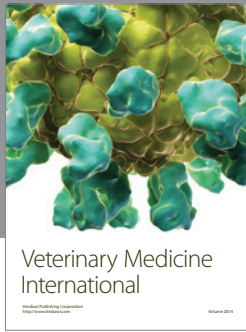
Acknowledgments

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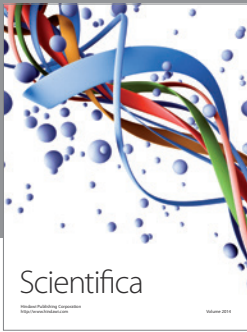
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