Acta Societatis Botanicorum Poloniae

DOI: 10.5586/asbp.3514

Publication history
Received: 2016-03-16
Accepted: 2016-09-07
Published: 2016-09-30

Handling editor
Beata Zagórska-Marek, Faculty of Biological Sciences, University of Wrocław, Poland

Authors' contributions
MK, DC: NRA measurements in the field; MK: laboratory analysis of NRA; JLK, MK, DC: measurements of NO_2; JLK: map of NO_2 producing; DC: data analyzing; DC, MK, JLK: writing manuscript

Funding
This study was financially supported by Institute of Environmental and Engineering Protection at University of Bielsko-Biała.

Competing interests
No competing interests have been declared.

Citation
Chmura D, Krywult M, Kozak JL. Nitrate reductase activity (NRA) in the invasive alien *Fallopia japonica*: seasonal variation, differences among habitats types, and comparison with native species. Acta Soc Bot Pol. 2016;85(3):3514. http://dx.doi.org/10.5586/asbp.3514

ORIGINAL RESEARCH PAPER

Nitrate reductase activity (NRA) in the invasive alien *Fallopia japonica*: seasonal variation, differences among habitats types, and comparison with native species

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Abstract

Nitrate reductase activity (NRA) was studied in the invasive alien plant *F. japonica* (Japanese knotweed) during the vegetation season and among natural, semi-natural, and human-made habitats and compared with NRA in selected native species. NRA was measured directly in the field from the beginning of May until the beginning of October. NRA was much higher than in the plant's native range, i.e., East Asia, and showed a high degree of variation over time with the highest values being reached at the stage of fast vegetative growth and at the beginning of fruiting. NRA was highest on dumping sites probably due to the high nitrogen input into soils and near traffic and the emission of NO_x by vehicles. A comparison of the enzyme activity in four selected native plant species indicated that NRA in *F. japonica* was the highest with the exception of *Urtica dioica*, which exhibited a similar activity of the enzyme. A detailed comparison with this species showed that differences between these species on particular dates were influenced by differences in the phenology of both plants. The initial results that were obtained suggest that nitrogen pollution in an environment can contribute to habitat invasibility and a high level of NRA, which in addition to the many plant traits that are commonly accepted as characteristic of invasiveness features, may be an important factor that enhances invasion success.

Keywords
nitrogen assimilation; plant invasiveness; *Reynoutria japonica*; knotweed

Introduction

Nitrogen fixation was pointed out by Rejmánek [1] who described the biological attributes of invasive alien species as one of possible factors that could explain species invasiveness. In this study, we focused on the nitrate reductase activity (NRA), which is responsible for the metabolism of nitrogen. We assumed that nitrogen use could be a significant factor in determining invasiveness because it enhances the growth and fecundity of plants. Although nitrogen is known as one of the most abundant biogens on Earth, it is widely known that there is a lack of this nutrient in many terrestrial ecosystems. This is one of the most important factors that limit plant growth [2,3]. Although free nitrogen may be fixed from the atmosphere by some free living and symbiotic prokaryotic organisms [4,5], this form of nitrogen is not available to vascular plant species. It is only available in the form of nitrate and/or ammonia, which may be absorbed by tissues from soil or from atmospheric fallout [6,7]. Soil nitrate and ammonia are believed to be the most important sources of available nitrogen for vascular plants, but gaseous pollutants such as nitrogen oxides, gaseous ammonia, nitric acid vapor, and also the nitrate that falls with atmospheric dust and is then directly absorbed by leaves may also influence the total pool of a plant's available nitrogen [8–10]. There is no doubt that invasive alien plants (IAS) were an important component of the environmental changes during the last century. It is generally accepted that biological invasions pose the second biggest threat to biodiversity after the loss or transformation of habitats [11,12]. Native species seldom threaten natural biodiversity; however, there are exceptions, e.g., *Brachypodium pinnatum* [13]. IAS have especially taken hold in areas that are situated close to intensive human movement and other human activities. It is often stated that one of the characteristics of successful plants is an ability to produce large numbers of diaspores and to grow to reproductive maturity as fast as possible during periods in which there is the greatest availability of resources [14–16]. The Japanese knotweed *Fallopia japonica* (Houtt.) Ronse Decraine (*Reynoutria japonica* Houtt.) of the Polygonaceae family is mentioned amongst the IUCN's 100 most invasive species worldwide [17]. Few studies have been devoted to its biochemical or ecophysiological aspects in terms of its invasiveness. Nevertheless, some biological plant traits on the ecophysiological level are crucial factors in explaining the behavior of plants. One such trait is a plant's ability for nitrogen uptake. Nitrogen availability is an essential limiting resource for plant growth and its efficient use may increase fitness. To the best of our knowledge, no previous studies have explored the NRA in *Fallopia japonica* in an adventive range. The only paper that was devoted to, among others, NRA in *F. japonica* was a paper by Tateno and Hirose [18] but it concerned the species as a pioneer plant on Mt. Fuji in its native range. Nitrate reductase (NR) is an enzyme that plays the key role in the nitrate fixation response to many environmental factors. In addition to the presence of a substrate (nitrate), the activity of the enzyme depends on many other factors such as temperature, the water status of the plant, the intensity of solar light, and others, and therefore its activity is determined by many variables [19]. On the other hand, using some of the methodology that has been developed, NRA may be a good indicator of the response of different plant species to the presence of enhanced nitrate species in the environment [20]. Diekman and Falkengren-Grerup [21] believe that NRA has a lower value of prediction of plant responses to atmospheric deposition and enhanced soil nitrogen levels; however, differences in nitrogen soil are a consequence of not only atmospheric deposition but also the decomposition of biomass including wastes (e.g., in ruderal habitats).

This study was done in order to assess the NRA in Japanese knotweed, which is an invasive species in southern Poland. Additionally, a comparison of enzyme activity on sites that are more and less affected by nitrogenous pollution should indicate whether *F. japonica* responds to higher nitrogen availability in the environment. Moreover, comparisons with selected native species can contribute to the knowledge about the higher competitiveness of *Fallopia japonica*.

The following questions guided our research:

- Is NRA higher in *F. japonica* in an alien range than in a native one?
- When is the activity of NR the highest during the vegetation season?
- Are there any variations in NR activity among sites due to the type of habitat and air pollution?
- Is the activity of NR in *Fallopia japonica* higher when compared to native species including nitrophilous species?

Material and methods

The research was conducted in the Bielsko-Biała region of Upper Silesia (Cieszyn Hills), which is situated in southern Poland. The list of localities with *F. japonica* populations is given in Tab. 1. In general, *F. japonica* is more abundant and invasive in the southern part of the country, especially in the aforementioned region [22,23]. As previous studies have shown [24,25], the species is chiefly confined to wetlands and anthropogenic habitats such as railways and urban wastelands (roadsides, parks,

Tab. 1 List of sites with *Fallopia japonica* populations, geographic coordinates of towns and type of habitat.

No.	Locality of population	Latitude (N)	Longitude (E)	Habitat
1	Bielsko-Biała, thickets	49°50′	19°04′	Roadside
2	Bielsko-Biała, near Biała River			Riparian
3	Bielsko-Biała, dumping site (1)			Dump
4	Bielsko-Biała, dumping site (2)			Dump
5	Bielsko-Biała, Żwirki i Wigury square			Roadside
6	Bielsko-Biała, Straconka, thickets			Roadside
7	Bielsko-Biała, Wapienica, thickets			Roadside
8	Cieszyn, meadow	49°45′	18°38′	Roadside
9	Porąbka, near dam	49°49′	19°13′	Riparian
10	Porąbka, site near bridge			Riparian
11	Kozy, meadow	49°51′	19°09′	Roadside
12	Korbielów, stream near Pilsko Mt.	49°34′	19°21′0″	Riparian

gardens, etc.). Nitrate reductase activity (NRA) is typically assayed in vivo by measuring nitrite production in tissue that has been vacuum infiltrated with a buffered nitrate solution [26]. For this study, a nitrate reductase assay was adapted from a number of studies [6,26] with our own modifications [27]. The sampling and measurements were carried out in similar weather conditions, i.e., only on sunny days between the hours of 11 a.m. and 1 p.m. solar time.

In order to avoid water stress and to minimize tissue damage, circles 3 mm in diameter were cut using a hole punch and placed into test tubes immediately after collection. The leaf tissue was then subjected to vacuum infiltration (with a manually operated vacuum pump) at 0.33 atm. for 5 minutes and incubated in the buffer for 2 hours at 20°C in the dark. The incubation buffer contained 0.1 M KNO_3, 0.1 M K_2HPO_4, and 0.6% 1-propanol and was adjusted to pH 7.0 using HCL and KOH. Four replicates of leaf tissue were collected in one sample. One replicate (test tube) contained 15–20 circles cut from at least 10 shoots of *F. japonica*. For the temporal study of the variation in NRA on one site, an area of Bielsko-Biała near the Białka River was chosen and field measurements were done seven times per vegetation season (from May until the middle of October; Tab. 2). The temporal variation of NRA was analyzed per population but not per developmental stage in order to better reflect the actual field conditions. Due to differences in the developmental stages of the plants among the sampling dates, the proportions of the leaf tissue from vegetative, flowering, fruiting, and senescent stems were different, depending on the contribution of a particular type of shoot in the stand. In the end, the number of test tubes containing leaves from particular developmental stages changed over the course of the investigation, according to the scheme presented in Tab. 2.

Tab. 2 Dates of sampling and the number of replicates in a sample of plant tissue from various types of shoots in the temporal study of nitrate reductase in *Fallopia japonica* leaves.

Code of series	Date	Shoots				*n*
		vegetative	flowering	fruiting	senescent	
I	08.05.2013	4				4
II	29.05.2013	4	-	-	-	4
III	19.06.2013	4	-	-	-	4
IV	24.07.2013	4	-	-	-	4
V	28.08.2013	1	2	1	-	4
VI	24.09.2013	-	2	2	-	4
VII	12.10.2013	-	-	-	4	4

For the spatial comparison of NRA, 12 sites in the Cieszyn Hills and the adjacent region were randomly selected in 2013 (Tab. 1). Each site was situated in a river valley on alluvial soils in urban, suburban, and rural areas but had slightly different conditions in terms of the population. In order to avoid biases due to differences in the developmental stages among the investigated sites, only flowering shoots were taken into account. Four replicates per a stand were collected. The type of habitat was assigned as riparian (natural type), roadside (thickets in the vicinity of roads – semi-natural type), and dump (dumping site – anthropogenic type).

Two procedures were applied in order to compare *F. japonica* NRA with native species. In each procedure, four replicates per species were collected at each site. The first procedure included a comparison of NRA among Japanese knotweed and four native herbs: *Galeopsis pubescens* Besser, *Impatiens noli-tangere* L., *Urtica dioica* L., and tree *Quercus robur* L. All of these species were present at site No. 6 on which thickets grow close to the road (Tab. 1). The leaves that represented a similar developmental stage, i.e., flowering shoots, were taken for analysis. This sampling was conducted in the period from May (flowering of *Q. robur*) until the beginning of July (flowering of *I. noli-tangere*). In the second procedure, performed in the next year 2014, in which NRA between *F. japonica* and native nitrophilous species – *Urtica dioica* was compared, four different sites: 1, 2, 6, and 7 (Tab. 1) were used in order to examine how site and time affect the differences in NRA between the two species. The field measurements were taken in five time series: the beginning of May, June and July, the end of August, and in October.

In order to examine the relationship between the concentrations of air nitrogen and NRA in *Fallopia japonica*, a determination of the nitrogen dioxide in the air, but only in the area of Bielsko-Biała, was performed. The estimation of the spatial variation of nitrogen dioxide (NO_2) was done using the method by Amaya–Sugiura [28] as modified by Krochmal and Górski [29]. This method uses the passive exposure of the absorbing material. In order to ensure the accuracy of the measurements in each of locality, three passive samples were exposed, whereas the value of the blind study was calculated based on additional unexposed samples. The final result was taken as the arithmetic mean of the values from the three samples, but in the cases in which the values differed by 25%, the mean was then rejected. All of the laboratory determinations were conducted in the laboratory of the Cracow University of Technology. Prior to the measurements in the field, a total of 56 localities that were situated in the entire area of Bielsko-Biala were selected. The monthly means for the concentrations of NO_2 (mg/m^3) during the vegetation season of 2013 were calculated based on the field measurements. In the final step, a map of the nitrogen dioxide content was created using Surfer software.

To test the significance of the differences among the dates when measurements were taken during the vegetation season, Welch's ANOVA followed by the Tukey test were applied for multiple comparisons. In order to avoid any autocorrelation due to the time of each measurement date, shoots of *Fallopia japonica* were randomly sampled. The normality of distribution and homogeneity of variance were checked using the Shapiro–Wilk test and the Levene test, respectively. The Spearman rank correlation test was used to examine the relation between NRA in *F. japonica* and the concentration of NO_2. For the purpose of this analysis, measurements of NRA were conducted once again at the seven sites in the vicinity of Bielsko-Biała. All of the statistics were done using the free R software [30].

Results

NRA was extremely variable during the vegetation season (Fig. 1, Tab. 3). The highest activity was recorded in May and at the end of September when some shoots were still in the blooming phase while others were fruiting (Fig. 1, Tab. 3). NRA slowed down when almost all of the shoots were fruiting. The activity of nitrate reductase showed significant variations among the sites that were studied (Fig. 1, Tab. 3). The highest mean values of NRA were observed on dumping sites and the lowest in natural riparian biotopes and wastelands near roads (Fig. 1).

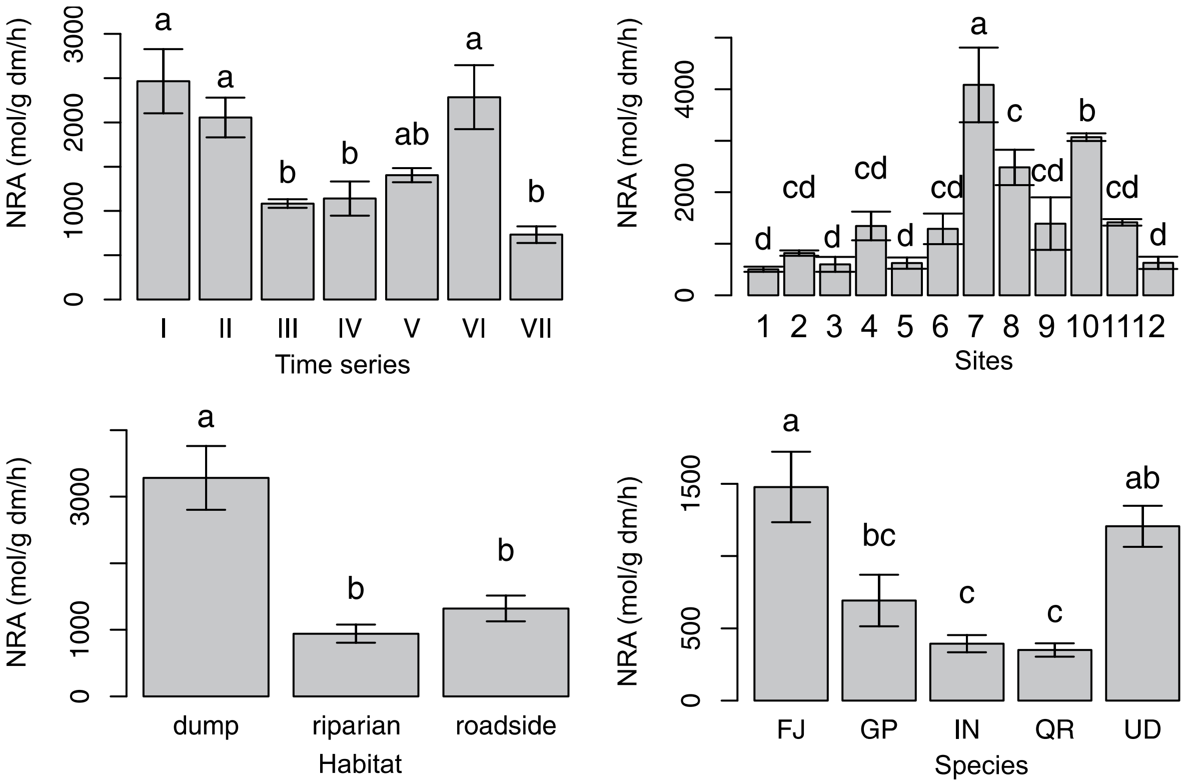

Fig. 1 Temporal variation (time series) and spatial (sites and habitat type) of nitrate reductase activity (NRA) in *Fallopia japonica* (FJ) leaves and comparison with native species: *Galeopsis pubescens* (GP), *Impatiens noli-tangere* (IN), *Quercus robur* (QR), *Urtica dioica* (UD). Means ±*SE* are presented. For codes of time series and sites, see Tab. 2. Different letters above bars indicate that the mean values differ significantly at $p < 0.05$

Tab. 3 Univariate ANOVA* or Kruskal–Wallis test# of the effect of species, time and site on nitrate reductase activity.

Effect	*df*	Statistics	*p*
Temporal and spatial variation in *F. japonica*			
Time#	6	23.58	<0.001
Site#	11	36.14	<0.001
Habitat#	2	15.75	<0.0001
Comparison of NRA among four native species and *F. japonica*			
Species#	4	46.45	<0.0001
The effect of species (*Urtica dioica* vs. *F. japonica*) and site and time*			
Species	1	0.04	0.8368
Site	2	0.45	0.6387
Time	10	7.73	<0.001
Species × Site	2	1.80	0.1685
Species × Time	9	9.37	<0.001
Site × Time	1	1.22	0.2697
Species × Time × Site	1	0.85	0.3593

The comparison of NRA in alien species to that in native species revealed significant differences (Tab. 3) among the species for which the highest values of NRA were found in *F. japonica* and *Urtica dioica*. In the latter, the mean total NRA was a little lower but the difference was non-significant. Further analyses between *F. japonica* and *U. dioica* from four different sites and in five time series demonstrated that only time and interaction of time and species was significant. At some sites only knotweed plants survived in the fifth time series (Tab. 4). There was a high positive correlation ($r_s = 0.89$, $p < 0.001$) between the NRA in *F. japonica* and the concentration of NO_2 in air (Fig. 2).

Discussion

The results obtained during this study show that *Fallopia japonica* exhibited high levels of nitrate reductase activity on all of the sites that were studied. There was also a high degree of the diversity in the activity of this enzyme among the sites that were examined. The highest values of NRA, as high as 5000 nmol per g of dry mass per hour, occurred on the dump sites in Lipnik as well as seasonally during the spring and the late summer in the city center of Bielsko-Biała (Fig. 1). The values of NRA in *F. japonica* that were reported previously [18] demonstrated that the activity of this enzyme was many times lower in its natural range and habitat. Obviously, it is not possible to directly compare those results with each other. The

Tab. 4 Comparison of NRA (mol/g dm/h) between *F. japonica* (F) and *Urtica dioica* (U) in time at four sites.

	Site 1 (thickets)	Site 2 (riparian)	Site 3 (forest margin)	Site 4 (thickets)
I(F)	2465.5 ±724.7 ^{ab}	3006.8 ±469 ^{a}	1191.8 ±1102.6 ^{bc}	1647.6 ±520.9 ^{bc}
I(U)	1892.8 ±1589.1 ^{bcd}	2726.5 ±618 ^{ab}	3468.0 ±1090.6 ^{a}	1672.5 ±441.6 ^{bc}
II(F)	3077.5 ±540.6 ^{a}	1124.0 ±319 ^{de}	1084.0 ±164.7 ^{bc}	1140.8 ±610.5 ^{bcd}
II(U)	946.0 ±174.9 ^{d}	649.0 ±90.7 ^{e}	776.3 ±148.3 ^{bc}	821.9 ±368.1 ^{cd}
III(F)	1083.0 ±97.6 ^{d}	2873.5 ±1690 ^{ab}	605.8 ±71.4 ^{c}	1257.3 ±1131.7 ^{bcd}
III(U)	945.5 ±128.3 ^{d}	2179.8 ±694 ^{abc}	570.3 ±64.1 ^{c}	994.7 ±642.0 ^{bcd}
IV(F)	1404.0 ±161.0 ^{cd}	733.8 ±35.8 ^{e}	1414.8 ±124.2 ^{b}	3312.8 ±1382.4 ^{a}
IV(U)	2067.3 ±433.5 ^{bc}	1889.5 ±409 ^{bcd}	1495.3 ±533.4 ^{b}	1734.0 ±664.9 ^{b}
V(F)	-	1639.5 ±543 ^{cd}	455.5 ±29.0 ^{c}	732.1 ±295.9 ^{d}
V(U)	-	-	-	3742.0 ±2340.0 ^{a}

The values with the same letters within a column do not differ significantly at $p < 0.05$ (Kruskal–Wallis test followed by Conover test). Time series: I – beginning of May; II – beginning of June; III – beginning of July; IV – the end of August; V – October.

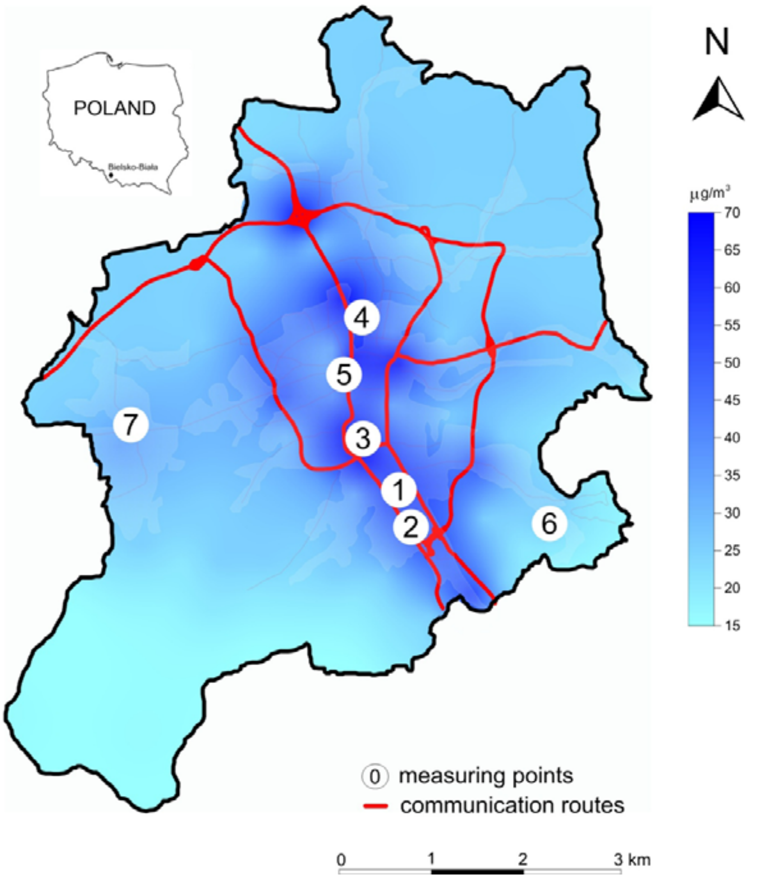

Fig. 2 The spatial distribution of NO_2 concentration (immission) in Bielsko-Biała in 2014.

altitude of the site was much higher, the character of soils was different, and the content of nitrogen compounds in the atmosphere probably differed significantly. The climatic conditions were also probably completely different here. On the other hand, we also have to consider some differences in many of the details in the methodologies that were used in these experiments, which may also have influenced the results that were obtained [18,31]. There was no possibility to do comparative studies on NRA in a native range using the present methods; however, some light can be shed by a comparison with native species. As was exhibited, selected native plants revealed lower NRA. The species that had a comparable activity of NRA was *Urtica dioica*. There is one study in the literature that demonstrated the activity of NRA that was measured in over 100 angiosperm plants in the field [32]. Woodland, fringe, and ruderal plants were studied in that research. The methods used in that study were different and thus the results cannot be compared; however, the trend is similar, i.e., *Urtica dioica* also exhibited the highest NRA. Generally, in our study there were no significant differences between *F. japonica* and *U. dioica* but detailed research demonstrated that time and site affected the differences. According to Bímová et al. [33], Tokarska-Guzik et al. [34], and Chmura et al. [35], the nettle *U. dioica* is one of the few species that is capable of coexisting in *Fallopia* stands. As far as the time is concerned, it has been known that NRA can vary during ontogeny (phenology) in plants since Andrews [36]. It is not clear whether or not NRA is affected by the concentration of nitrogen in the soil. Studies have shown either a positive relationship, i.e., increasing NRA with the N concentration in the soil [36] or no dependence but only species-level differences [37]. The other possible explanation is the deposition of atmospheric nitrogen. It was found that nitrate reductase activity is significantly correlated with the nitrogen compounds that are dry deposited on *Pinus ponderosa* Dougl. ex Laws. needles in the San Bernardino Mountains, California, an area that is affected by traffic pollution from the Los Angeles basin [9]. In the present study, we confirmed this phenomenon by the correlation between the NO_2 in the air and NRA in *F. japonica* leaves. There is also evidence of the strong impact of motor vehicle traffic on the deposition of nitrogen compounds. The additional N deposition that is attributable to vehicle exhaust gases is between 1–15 kg N ha/y depending on traffic density [38].

During other research that was conducted by the authors in the past, only one of the investigated species demonstrated activity similar to that of *F. japonica*. Endemic *Begonia crateris* Exell. (Begoniaceae) in a tropical forest on St. Thomas Island showed a high and constant activity of nitrate reductase [39]. It is an interesting fact that the other species from this habitat that were studied demonstrated a low or no NR activity. Moreover, the concentration of nitrogen compounds and the atmospheric fallout was low in that environment [39]. Some plants can use quite different sources of available nitrogen forms [40]. They can also demonstrate different adaptations to low, moderate, and high concentrations of nitrogen forms in the environment. That phenomenon was found for *Deschampsia antarctica* Desv. [41]. However, because the nitrate reductase enzyme is characterized by a rapid turnover and a sensitivity to many abiotic factors such as light intensity, water stress, the influence of other chemical compounds [6], and ultraviolet radiation [31], it may be a good indicator for monitoring the physiological condition of plants as well as the saturation of nitrogen in an environment. The results obtained during these preliminary studies seem to be interesting enough to indicate that more attention should be paid to this plant as a model invasive species that may also give important information about the different forms of nitrogen in an ecosystem. NRA was probably the highest on dumping sites due to a high nitrogen input into soils as well as in areas that were near traffic and the emission of NO_x by vehicles. Interest in biomonitoring and ecological studies has grown due in recent decades due to the increase in the pollution of atmospheric air by nitrogen compounds. Nitrogen oxides (NO_x) are a major component of urban air pollution. The nitric oxide (NO) that is emitted from the combustion of fossil fuels is rapidly converted to nitrogen dioxide (NO_2) in the air [14]. It is possible that nitrogen pollution can contribute to the spread of *Fallopia japonica* in urban and suburban areas. In the case of this species, it seems that nitrogen pollution that is due to the deposition of atmospheric nitrogen or perhaps due to the ruderalization of habitats may be the cause of the higher invasibility of certain habitats, i.e., the vulnerability of the habitats to invasions.

To sum up, we can conclude that *Fallopia japonica*, which is an invasive plant originating from Eastern Asia, demonstrated high nitrate reductase activity in all of the sites investigated in southern Poland. The seasonal activity of nitrate reductase in *F. japonica* leaves changed during the vegetation season and reached two maximum values in the spring and late summer, which may be connected with a shift in the allocation of the biomass, the development of the leaf canopy as well as flowering and fruiting, respectively [34]. A comparison of NRA in alien species with that in native plant species confirmed that the enzyme activity in the species that were studied is very high. Future research should take into account analyses of total nitrogen in the plant tissues and the impact of the atmospheric deposition of nitrogen. The initial results that were obtained in this study suggest that, apart from the many plant traits that are commonly accepted as characteristic invasiveness features, high NRA could be an important factor that enhances the invasion success of alien species. An analysis of NRA could possibly be useful in predicting the rate of nitrogen assimilation among habitats and among different invasive alien and native species and to test the theory of fluctuating resources sensu Davies et al. [42]. Monitoring the deposition of atmospheric nitrogen may permit questions about whether changes in the level of nitrogen oxides influence the abundance of knotweeds in some habitats to be answered.

Acknowledgments

We would like to thank Magdalena Pastuszak and Beata Konior for their technical support in the field. Michele Simmons kindly improved language of the paper.

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