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Interaction of Two Neonicotinoid Insecticides and *Lr* Genes Focusing Wheat Growth and Residues

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Abstract

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Seedlings of ten Lr near-isogenic lines (NIL) and four wheat lines with enhanced resistance to *Puccinia triticina* were treated with overdosed neonicotinoid insecticides. The enzyme of *Lr 20* gene accelerated thiacloprid degradation while *Lr 29* gene accelerated thiamethoxam degradation according to NILs upper plant parts lengths reduction by 6% or 10% six days after the last of three daily treatments. Lr 27 and Lr 33 effects were intermediate on thiamethoxam but only in the driest conditions. Among other NILs the growth was increased 1–5% by N faster release of S or Cl. The accumulation of Lr 20 and Lr 9 facilitated treatment in the late grain filling period when the amount of their circled residues in seed was permissible. In semiarid regions, when thiamethoxam was applied before June, respecting the adequate leaf area duration of prevalent varieties, interaction with Lr 29 could be also practical through simultaneous release of fungi reducing elements.

Keywords: insecticide residues; wheat protection; *Lr 20; Lr 29*

The global status of sixty-nine discovered wheat leaf rust (*Lr*) reducing genes was presented by Huerta Espino *et al.* (2011). They were divided into two groups according to specific or nonspecific impact on the parasite population (Nelson 1978). Next clustering was according to the growth stage when a hypersensitive reaction was recognised (Wanishe & Milus 2004). Early abortion of the parasite (Anker & Niks 2000) and absence of appressorium formation within for one hour wet conditions at optimal temperatures (De Vallavieille-Pope *et al.* 1995) suggested water deficiency as base of nonspecific resistance. Across the recognised growth differences in NILs at seedling and adult stage those genes were classified as ABC transporters (Krattinger *et al.* 2009) or starch degrading enzymes. Race specific *Lr* genes had an influence on the accelerated degradation of proteins stored in wheat seed. Their optimal conditions and location had to be different from PDI enzymes (Ciaffi *et al.* 1999) while Lr 21 cloning (Huang *et al.* 2003) facilitated the first classification in endoprotease family by Jerković *et al.* (2013a). Race specific *Lr 16* and *Lr 29* genes were recognised as responsible for accelerated cleaving of dithiocarbamate and phthalamide fungicides through reduced seedling growth (Jerković & Prijić 2012). A hypothesis was formulated that some of the specific *Lr* genes could accelerate the release of sulphur from neonicotinoids with the same consequences. By other strategies based on pesticide modification or gene evaluation for their inhibition (Castle *et al.* 2004) they were transformed or linked with protein residues in seed found to be reversible according to the Test Biothech report from 2013.

The aphid population on seedlings was increased by soil fertilising but still low frequented (Praslička & Miština 2004) while studies of dynamics in spring (Malschi 2003) confirmed a transparent problem of non-synchronised certainly rentable and safe food consequential treatments. Thiamethoxam was found to be useful for aphid reduction (Miao *et al.* 2014) as well as a contact effect on some Coleoptera larvae was recognised (Laznik *et al.* 2010). Problematic was the protection from *Puccinia triticina.* A forecasting model for parasite development depended on data collected during the last week of May in a region where grain filling of adequate wheat varieties finished from 15th to 25th of June (Jerković *et al.* 2012b). The time for degradation of neonicotinoid insecticides containing S incorporated into different pentagonal structure was proposed to be around three weeks while for most of

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the fungicides besides the afore-mentioned ones it was even longer (EPA SLN No. CO-090004; Osborne & Staine 2009). The residual structures of some insecticides stored in seed were recognised after six months (Mensah *et al*. 1979). The same but faster effect on fungi was expected according to a faster release of active elements in phthalamide-based fungicides. Enhanced resistance to *Puccinia triticina* when glyphosate was applied and impact of *Lr* genes at mentioned situation was reported by Anderson and Kolmer (2005). Such enzymatic degradation of neonicotinoids was found as a possible solution to the above-mentioned three practical problems at once.

MATERIAL AND METHODS

Different specific *Lr* genes introduced into the Thatcher background after backcrossing by substituted chromosomes (Lr NIL) were selected for the testing on the basis of adequacy for gluten (Lr 1) or different fungicides accelerated degradation as were Lr 16 and Lr 29, indicated necessary accumulation with nonspecific genes for expression (Lr 27+Lr31, Lr 33+Lr 34) as well as effect on the local parasite population reduction (Lr 9, Lr 20, and Lr 24). As nonspecific or adult plant resistance controlled representatives Lr 22b, the most common in discussed Lr lines and determined also in Thatcher, and Lr 2c were added.

An experiment was conducted in the Institute of Field and Vegetable Crops, Rimski Šančevi, Novi Sad, Serbia (45°33'N, 19°85'E, 82 m altitude). Lines were grown in the glasshouse at an average air temperature of around 20°C under day/night regimen of 10/14 hours. Lr NILs were simultaneously sown in pots (30 × 25 × 15 cm) while density was approximately one plant per 50 mm^2. Daily watering (drinking-quality water) of the soil was stopped two days before insecticides were applied. Twenty plants of each NIL line with nearly equal lengths were selected six days after germination. In a subsequent trial approximately 50 seeds were sown randomly in pots (5 × 5 × 7 cm). Simultaneous treatments with one of the insecticides based on thiacloprid ($C_{10}H_9ClN_4S$) and thiamethoxam ($C_8H_{10}ClN_50_3S$) were applied on half of NILs. The solution containing 0.5 g or 0.5 ml of insecticides and 0.5 l of water in the amount of 100 ml was applied in triple on approximately 1 m^2, daily sequenced, using hand sprayer. After 2 h the plants were treated with the same amount of water. The estimation of plant part lengths was performed six days after the last treatment. For further calculations a criterion was used that the stems of the NIL sample could be different in the interval of 5 mm. NILs plants were also chosen according to LGR values (LGR = the sum of the length of the first leaf and stem divided by the length of the second leaf) where differences had to be in 0.1 interval. At least five plants out of the twenty viable in each of the replications had to be in a representative sample for the calculation of average part lengths of the NILs. Differences in stem lengths of the control and treated NILs, its correlation with assumed plant length was estimated. The representative sample was six plants of five control NILs as well as randomly chosen fifty plants from that part. Lines in F_5 generation selected from the progenies of the crosses NS 1772 (Lr 19) and NS 1775 (Lr 2a) with Lr 20, Lr 21, and Lr 29 NILs were tested using *Puccinia triticina* monopustule isolate described by virulence at Lr 1, Lr 2a, Lr 9, Lr 19, Lr 20, Lr 24, and Lr 29 NILs, which prolonged latency period (LP) by approximately one day, following the procedure described by Jerković and Putnik-Delić (2004). Lines were grown in 0.2 l pots and treated twice with thiamethoxam while in all other cases the trial was equal to the afore-mentioned one.

RESULTS

Treated with thiacloprid, the growth of the Lr 20 NIL was maximally reduced while focusing on Lr 29 NIL a decrease of 3% was observed in both sowing regimes. Its LGR values of treated and control parts were close to the difference of Lr 20 NIL. When NILs were grown in larger pots, LGR was relatively maximally increased while no positive effect of insecticide was found out. In higher density, according to LGR and growth average decrease, the positive effects were increased by up to 5% (Table 1).

Focusing on the afore-mentioned two genes, when thiamethoxam was applied, the effects were oppositely ranked while by Lr 27 and Lr 33 they were intermediate but only when LGR and growth of adequate NILs were relatively maximally decreased. If it was opposite, their degradation ability had been lost whereas positive effects were similar to other NILs (Table 1).

The stem and sum of all part lengths were very strongly correlated (r = 0.89–0.94). The average differences in stem lengths between treatments and control were below 4% in 88% of NILs when all trials were focused. There was no possibility to choose enough plants respecting previously mentioned criteria

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Table 1. Influence of thiacloprid on growth of Lr NILs seedlings

Lr NIL		Length (mm)			Average upper ground length (T/K) × 100	LGR	Length (mm)			Average upper ground length (T/K) × 100	LGR
		stem in 0.2 l pots	1st leaf	2nd leaf			stem in 5 l pots	1st leaf	2nd leaf		
Lr 1	K	53.5	148.8	128.5		1.57	48.8	110.5	140.1		1.14
	T	53.0	147.2	127.0	100	1.58	50.1	114.2	143.4	103	1.16
Lr 9	K	55.6	139.0	156.7		1.16	54.3	118.5	139.9		1.23
	T	55.0	139.2	150.5	100	1.20	51.5	103.4	134.7	102	1.15
Lr 16	K	53.0	133.0	140.2		1.33	45.5	98.1	125.5		1.15
	T	54.0	133.8	143.5	100	1.31	50.1	105.3	132.3	105	1.17
Lr 20	K	54.0	129.3	141.6		1.29	48.0	110.6	130.3		1.22
	T	53.0	118.6	136.2	96	1.26	48.9	99.1	124.5	94	1.19
Lr 24	K	54.5	129.4	146.7		1.25	44.6	97.8	126.5		1.13
	T	53.6	129.2	140.3	100	1.30	41.6	95.8	119.9	102	1.15
Lr 27	K	52.2	136.2	141.9		1.33	47.7	105.1	132.3		1.15
	T	51.5	134.4	139.9	100	1.33	47.2	104.8	138.7	102	1.10
Lr 29	K	50.2	122.0	131.0		1.33	38.1	86.8	91.1		1.37
	T	50.7	120.6	121.1	97	1.42	53.3	110.5	113.6	97	1.44
Lr 33	K	39.0	106.5	120.0		1.30	44.0	96.6	122.3		1.18
	T	40.0	109.2	125.0	100	1.28	44.6	108.0	130.3	104	1.20
Lr2c	K	53.4	123.0	162.1		1.09	47.5	104.0	131.5		1.15
	T	54.4	124.0	163.2	100	1.09	46.7	103.3	132.0	102	1.14
Lr 22b	K	59.8	126.8	174.2		1.07	54.7	87.8	182.0		0.78
	T	62.2	130.2	180.2	100	1.07	54.2	84.3	185.5	101	0.75
Average upper ground length		52.6	129.0	143.9			48.1	102.3	133.8		

K – control NIL; T – treated NIL; LGR – sum of the length of the 1st leaf and stem divided by the length of the 2nd leaf

to complete both necessary trial parts. Furthermore, equal by stem length NILs carrying specific *Lr* genes were compared with those carrying Lr 22b or Lr 2c. The primary leaf length of the former appeared to be prolonged by 25% or 8% on average. SD of stem lengths in the trial was maximally 4.67 while when one of the extreme NIL was excluded, they were in the interval 2.27–3.19 mm. When Lr 29 and Lr 9 NIL were compared according to plant parts length the observed effect was below 1%. By comparison of Lr 9 and Lr 22b NIL controls in the trial when thiacloprid was applied, the largest difference in growth (4%) was found out. Average sums of NILs lengths of four trials calculated when insecticide effects were excluded were also compared, while differences between all six pairs were below 3%. Data when NILs were treated with thiacloprid and grown in larger pots were most critical, while if excluding the Lr 29 NIL result, the differences were below 2% so the limit of a possible error was defined when the effects were differentiated.

In the subsequent trial when the lines contained Lr 20 or Lr 29 with addition of other specific ones necessary for reaction type (RT) decrease to *Puccinia triticina* isolate, the growth was reduced by thiamethoxam approximately proportionally to the relative decrease of dose while positive effects were near those in previously described trials. When adequate NILs as parents were replaced by Lr 21, the effect on growth was positive like on NS 1772 × Lr 29 NIL. The LGRs of the lines were generally decreased in comparison with the trial when NILs were tested, the largest decrease was from the cross NS 1775 × Lr 29 (Table 3).

DISCUSSION

The amplified growth when fungicides were applied was related to additional foliar nutrition by released nitrogen or its units because the uptake of nutrients from soil in the discussed developmental

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Table 2. Influence of thiametoxam on growth of Lr NILs seedlings

Lr NIL		Length (mm)			Average upper ground length (T/K) × 100	LGR	Length (mm)			Average upper ground length (T/K) × 100	LGR
		stem in 0.2 l pots	1st leaf	2nd leaf			stem in 5 l pots	1st leaf	2nd leaf		
Lr 1	K	57.8	113.6	156.5		1.09	50.0	105.0	138.5		1.12
	T	61.4	122.6	171.0	102	1.08	51.1	104.4	152.7	103	1.02
Lr 9	K	53.7	121.7	153.1		1.15	54.3	118.1	145.2		1.19
	T	57.5	132.8	174.1	104	1.09	51.5	114.7	141.6	102	1.17
Lr 16	K	41.2	100.6	109.0		1.29	49.0	107.5	125.0		1.25
	T	43.0	101.0	116.0	101	1.25	46.3	98.7	117.0	102	1.24
Lr 20	K	57.2	126.8	132.1		1.39	50.6	106.7	127.0		1.24
	T	56.6	127.5	123.0	98	1.49	54.4	105.7	129.7	95	1.23
Lr 24	K	58.3	124.4	144.4		1.26	55.7	111.7	138.7		1.21
	T	53.3	116.3	139.3	102	1.22	53.8	116.2	139.5	103	1.21
Lr 27	K	57.7	126.5	139.0		1.35	53.4	110.9	157.1		1.04
	T	53.6	129.0	124.8	102	1.47	54.9	111.5	141.8	94	1.17
Lr 29	K	31.4	67.0	65.5		1.50	36.0	87.2	96.4		1.27
	T	24.5	63.2	44.4	98	1.99	51.3	86.1	118.8	90	1.17
Lr 33	K	40.5	91.3	104.5		1.27	47.4	93.2	146.5		0.96
	T	42.5	95.6	115.0	102	1.21	50.7	96.1	149.0	95	0.98
Lr2c	K	42.1	111.4	115.8		1.20	47.8	97.0	125.6		1.15
	T	43.6	115.6	119.0	101	1.32	52.0	110.0	145.4	103	1.14
Lr 22b	K	58.9	118.9	168.6		1.08	59.6	101.8	181.7		0.83
	T	59.3	118.4	175.8	102	1.04	59.9	102.6	186.5	101	0.82
Average upper ground lengths		49.7	111.2	129.5			51.5	104.3	140.2		

K – control NIL; T – treated NIL; LGR – sum of the length of the 1st leaf and stem divided by the length of the 2nd leaf

stage was not observed (von Vuurde & Tonneyck 1978). Nitrogen oxides behind vigour were first suggested by Mr. Denis Hamilton from Primary Industries and Fishers confirming the entry ability of insecticide structure or its external substitute to dry seed. Seedlings vigour is a result of unstable N acids during photosynthesis and OH relist able opposite to S and Cl (Ammann *et al*. 2005). The lack of positive effect like in other trials was related to wetter conditions and thiacloprid structure facilitating the simultaneous release of one N and a certain amount of Cl differentiate from thiamethoxam with N_2O.

Table 3. Influence of thiametoxam on seedlings growth of lines from NS and Lr NILs crosses

Combination	RT		Length (mm)			Average upper ground length (T/K) × 100	L GR
			stem	1st leaf	2nd leaf		
NS 1772 × Lr 20	0;	T	53.4	111.8	174.4	99	0.94
		K	49.0	110.2	154.4		1.03
NS 1772 × Lr 21	4	T	48.6	99.2	164.4	102	0.90
		K	39.0	104.4	112.6		1.27
NS 1772 × Lr 29	4	T	42.2	111.2	153.0	103	1.00
		K	43.8	118.8	163.8		0.99
NS 1775 × Lr 29	0;	T	36.5	95.6	116.8	97	1.14
		K	40.3	102.0	122.0		1.17

RT – reaction type; K – control NIL; T – treated NIL; LGR – sum of the length of the 1st leaf and stem divided by the length of the 2nd leaf

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When the influences of Lr 16 and Lr 29 on dithiocarbamate and phthalamide were compared according to the fungicide potential for forming Mg salts, the ratio of involved elements was correlated with decreased growth. In the recent study, when thiamethoxam was applied to Lr 29 NIL and positive effects were accounted, it could be assumed that growth was even more decreased while appearance was related to different entering ability and molecular weights of pesticides. However, in a phthalamide-based fungicide the N-S-C linkage was weaker than C-S-C after external Cl relist. Externally linked S and C molecular weight indicated the extended approach ability of Lr 29 product in comparison with Lr 16 adequate for S in dithiocarbamate. When recent results were focused, Lr 29 should release Cl from circles, while N was inhibited when there was not nearby S in the thiacloprid structure. Conditions adequate to facilitate the opposite to Lr 20 gene effect appeared to be more frequent while Cl hydrolytic release from thiamethoxam was behind the observed increased effect in comparison with Lr 29. The most transparent influence of Lr 29 on thiamethoxam degradation was related to drier conditions with a remark that sulphur linkages had to be cleaved at first and followed by nearby Cl ones immediately after. According to recently recognised characters, the Lr 29 product also appeared to be inadequate for strengthening the sulphur linkage cleaving, *vice versa* of Lr 20. Anyhow, according to LGR and growth of NILs reduced in drought conditions, Lr 27 or Lr 33 products had to be relatively the most hydrolytically unstable as well as recognised specificity to thiamethoxam could be occasional. There was no difference in hydrolytic stability between *Lr 20* and *Lr 29* genes. However, Cl appeared to be more constantly present while S had to be released at last. Besides S and most likely P, Cl was also targeted by *Lr* gene enzyme, suspicious when phthalamide was focused because of N-S-C-Cl_3 faster hydrolysis.

LGR of Lr 2c NIL in comparison with NILs containing specific *Lr* genes and Lr 22b was intermediate explained by the association with a complementary gene from nonspecific group of *Lr* genes according to the study when the effect of Lr Tc on 2D chromosome was defined (Jerković *et al.* 2013b). This gene in Lr 29 NIL most likely increased LGR. Of the additionally tested two lines enhanced resistance in comparison with Lr NILs was achieved by accumulation of specific *Lr* genes while *Lr 19* and *Lr 29* were not predicted because of location on 7D by review of McIntosh *et al.* (1995). The increased LGR of the progeny from NS 1775 × Lr 29 was due to replacing Lr 22b by Lr 2a. In the progeny from the cross of NS 1772 × Lr 29 NIL, Lr 2c had to be absent according to LGR. However, in agreement with other parasite-free investigation results, there was no complementary effect while it was confirmed simultaneously that these *Lr* genes were not from Lr 20 or Lr 29 cluster. When their products were accumulated, the most efficient insecticides degradation was expected. Initial molecular markers for *Lr* genes were reviewed by Cheikowski and Stepien (2001), for *Lr 20* by Neu *et al.* (2002), and for *Lr* 19 by Kassem *et al.* (2011).

For practical use of recent results the basic state was that covered grain, lack of continual wetting, and higher pressure of water in seed *vice versa* than during germination had to stop direct insecticide entry through the seed surface. For stopping the transfer of circled residua containing sulphur, chlorine or both in seed endosperm trough germ only combination of Lr 20 and Lr 29 has been proved as successful. In the last decade of May, depending on the variety of leaf area duration, respecting the time proposed for insecticide hydrolytic degradation, interaction of single Lr 29 and thiamethoxam was expected to be adequate for increasing the resistance to fungi across simultaneously accelerated active element release. However, enzymatic degradation of neonicotinoids was recognised as a possibility for reducing the three mentioned damage causers at once, when less than three weeks of green leaves area is preserved.

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