P 1 - Verrier - Chemical change / cigarette smoke mutagenicity associated with CMV / PVY infection in Burley tobacco

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Chemical change and cigarette smoke mutagenicity increase associated with CMV and PVY infection in Burley tobacco.

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Summary

It had previously been reported that challenging in field Burley tobacco varieties with CMV or PVY resulted in increased contents of total nitrogen and nitrate in cured leaves. In order to assess a possible impact of virus infection on smoke mutagenic effect, cigarettes were made with middle leaves from inoculated plants as well as from non-inoculated control plants of the same variety. Wet total particulate matter (WTPM) samples obtained from mechanical smoking were subjected to the Ames test using the TA98 *Salmonella typhimurium* strain, with S9 metabolic activation. Specific activity has been determined as being the linear regression slope of the revertant colonies count on WTPM concentration, and was expressed in number of revertant colonies per mg of WTPM. The specific activity was found to be higher in virus infected plants than in controls. Expressed in % of the control, these differences ranged from 0 to + 40 %. When challenged with PVY, varieties homozygous for the "va" deletion conferring resistace to PVY-N had lower specific activity increases than susceptible " Va " varieties. When challenged with CMV, all varieties showed higher specific activity than the controls (from +7 to +37 %). Co-inoculating a " Va " variety with CMV and PVY resulted in the highest specific activity (40% above the control). These results were obtained from two different field trials, respectively grown in 1998 and 1999. Effects of virus infestation showed the same trends in each year. Variation of the specific activity was positively correlated with total nitrogen and nitrate contents, and with the intensity of symptoms assessed from field scoring.

Introduction

It has often been recorded that changes induced by viral infestations in plants not only were restricted to yield losses, but also involved the chemical balances of the harvested organs or seeds, often with detrimental impact on qualitative traits. Works done in this area trace back as early as in 1913, when BUNZEL analysed sugar beets affected by the curly-top disease (1). In 1930, before the characterisation of the viral agents involved, DUNLAP confirmed that the C/N balance was affected by viral infestations in several crops, among which tobacco (2). By harvesting mature leaves on healthy and diseased plants growing side by side in field, he

could establish a general distinction between "mosaic diseases", where the C/N ratio is decreased, and "yellow diseases", where this ratio is substantially increased.

In 1948, HOLDEN and TRACEY(3) analysed healthy and TMV-inoculated greenhouse grown tobacco plants, and confirmed the results of DUNLAP for mosaic diseases: total amounts of N in both types of plants were the same, but dry matter accumulated in diseased plants was lower, resulting in a higher N / dry matter ratio.

Following the increasing knowledge of plant virus affecting tobacco, several works were conducted for studying the effect of virus on cured leaves, using field tobacco grown with the standard practices (4 to 12). Table 1 and 2 present data from these studies, which involve 6 species of single stranded, RNA + virus causing different types of mosaic in tobacco: TMV, TRSV, PVY, TEV, TVMV, and CMV.

Various virus and tobacco variety combinations were used, summarised in the 20 situations shown in table 2. The total nitrogen content of infested plants, when expressed in percent of the dry matter, is in every case but one higher than in healthy plants. When measured, the nitrate content is also substantially higher.

These differences were not statistically significant in every individual study. However, accumulation of data is strongly suggesting that single stranded, RNA + virus causing mosaic in tobacco have a profound effect on the leaf chemical balance, resulting in higher total nitrogen and nitrate contents.

In contrast, the total alkaloid content seems to be affected by some of these viruses, but not consistently in the same direction. In burley tobacco, PVY infestation decreased strongly the alkaloid content (5, 7, 8), however, in flue-cured, this resulted in an increase (11). TVMV decreased strongly the alkaloid content of burley tobacco (9). TMV, TEV and CMV did not seem to affect substantially this trait (10,12).

In three examples, mixed inoculations have been used. When compared to plants inoculated with only one virus, the importance of the chemical change is increased (7, 10, and 12). Two studies showed that tolerant or resistant varieties, defined as such according to symptom expression, were less influenced in their chemical composition by virus than susceptible varieties (9,11). In a previous work, we presented results obtained on Burley cultivars, which are consistent with all the above noted facts (13).

Since many compounds related to the health aspects of tobacco consumption contain nitrogen, it was of interest to check whether this increase in total N, despite not being connected with an increase in total alkaloids, would affect mutagenicity of the smoke.

Material and methods

Data presented here refer to two field trials, respectively grown in 1998 and 1999, and involving Burley varieties challenged with PVY and CMV (1998), or with PVY, CMV and CMV + PVY (1999).

1998 experiment

Tobacco varieties

Pedigrees and main traits of Kentucky 17 and TN86 have been published from their releases (14), (15). Kentucky 17 is highly susceptible to PVY. ITB 2204 is a F1 male-sterile hybrid, involving a VaVa female line (susceptible to PVY) crossed by a vava pollinator (resistant to PVY). Due to the recessive nature of the va gene, this hybrid is susceptible to PVY. ITB 218 is a male-sterile line, homozygous for the va gene. The va allele present in ITB 218 is inherited from VAM, therefore ITB 218 has non-secreting trichomes.

No known resistance factors to CMV are present in these four burley varieties.

Layout

A split-plot design with 4 replications was used. The 4 varieties were designed as main plots. Sub-plots in each main plot were devoted to the virus treatments (non-inoculated / inoculated with CMV / inoculated with PVY). Each sub plot consisted of one row of 27 plants. Distance between rows was 90 cm, and distance between plants in a row was 39 cm, for a plant population of 28500 plants / ha. Transplants were produced with the floating bed system, in a plastic greenhouse treated with insecticides for aphid control. It has been shown that in these conditions in France, viral contamination by aphids does not occur in greenhouses, but starts in field after transplanting. Transplanting was performed on May 18th, 1998 and at this date all transplants were free of virus symptoms.

The trial was planted in the same field as other burley trials. Standard fungicide treatments were applied. Insecticide treatments for aphid control were applied (June 5th and July 16th), however, this did not prevent completely viral contamination by aphids. Despite a low degree of natural contamination by aphids, a few plants of the non-inoculated plots developed viral symptoms, which could be attributed to CMV or PVY.

Virus strains

The PVY strain used for the experiment was from the pathotype 2 (17). Prior to the experiment, it was inoculated in a greenhouse, to a set of VaVa and vava varieties, in order to check that it was able to produce necrotic symptoms only on susceptible, VaVa varieties. Leaves from ITB30 (VaVa) with necrotic symptoms were used to produce the inoculum.

The CMV strain used for the experiment was collected from tobacco in Lot et Garonne (South-West of France) in 1994 and maintained in the laboratory on tobacco. In a greenhouse with a constant temperature of 22 °C, it produced mosaic symptoms on the variety ITB30, from which leaves were taken as source of inoculum.

Mechanical inoculation

The inoculum was obtained from infected leaves crushed in a mortar. For 1 g of leaves, the sap was diluted into 4 ml of a solution Na_2HPO_4 (0.3 mol. l^{-1}) + DIECA (2g.l^{-1}) + 0,3 mg carborundum. This solution was kept at low temperature (4°C) in a heat-insulated container brought in field. Inoculation was performed in field on June 11th. Two leaves /plant were gently rubbed with the solution.

ELISA controls, visual symptom notation

DAS-ELISA tests using polyclonal antibodies marketed by the company SANOFI were

performed on inoculated plants, one month after inoculation, and results confirmed the multiplication of the virus in these plants. On inoculated treatments, visual expression of symptoms was scored according to the scales shown in table 5, on a plant by plant basis, on August 11th.

Topping, harvesting and curing, leaf sampling

The trial was manually topped (16 leaves) on July 28th, and inhibited at the same date with TAMEX AG®. Stalk harvest was performed on August 26th. Border plants, from top and bottom of rows, were not harvested, so that 24 to 25 plants were harvested for each subplot. Plants were air-cured, then leaves were removed and grouped into 4 leaf positions. Each leaf position was separated into 4 classes according to quality: class 1 = best, 2=middle, 3 = low, 4 = no commercial value. The quality index has been computed for each position according to:

IQ =(100* weight of class 1 + 70 x weight of class 2 + 40 x weight of class 3) / total weight of the sample.

A global IQ has been computed for each subplot, by combining IQ's of each leaf position with their total weights.

In each subplot, a composite sample of leaves from the third leaf position (mediane leaves) was set aside for chemical analysis. Whole leaves were involved in this sample, comprising midrib. Remaining mediane leaves from the same treatments (variety x virus) where gathered across replications, in order to get enough material for making cigarettes.

Chemical analysis

Leaf samples were ground into a 500-µ powder. Dry matter has been determined from oven dehydration. Nitrate content was obtained through CORESTA method n° 36. Total alkaloid (as nicotine) was determined using CORESTA method n° 35. Total nitrogen was determined with the nitrogen combustion analyser and accessories Model FP-228 from *Leco Corporation*. Nornicotine was estimated with a colorimetric method (16).

Smoking tests

Cigarettes (diameter: 7,9 mm, length: 70 mm) were made from whole shredded leaves (including midrib), and a sample of cigarettes with a constant resistance to draught was selected for ISO 3308 smoking tests. For each subplot, two smoking tests were performed, yielding two smoke particulate matter samples. These were diluted into DMSO (10mg/ml) and stored at -80 °C.

Ames tests

For each total particulate matter sample, six doses were studied: 0.05, 0.1, 0.20, 0.25, and 0.30 mg/ Petri dish. Ames test has been performed using S9 metabolic activation and the TA98 strain of *Salmonella typhimurium* (21). Numbers of revertant colonies have been counted from each Petri dish, then the slope of the linear regression of these numbers on doses has been computed. This slope is expressed in number of revertant colonies / mg of smoke total particulate matter, and is referred hereafter as "specific activity". The data shown

are the means of the two specific activity estimates obtained from each smoke particulate matter sample.

1999 experiment

This experiment have been performed with the same goals, methods and procedures than the 1998 one. Only treatments (varieties, virus strains...) that have been different are specified below.

Tobacco varieties

ITB 501 is a male-sterile F1 hybrid released in France for burley tobacco production in 1998. It is homozygous for the va gene, and, therefore, is resistant to the most common strains of PVY. The female line of ITB 501 inherits its va allele from VAM, whereas the pollinator line inherits its va allele from TN86. Kentucky 17 is involved in the pedigrees of both the pollinator and the female lines. Similarly to TN86, ITB501 has secreting trichomes. It has no known resistance factor to CMV.

The other variety used in 1999 is Kentucky 17.

Field trial

The layout has been similar to 1998. The date of transplantation was May 26th; the inoculation was performed on June 22nd, topping on August 4th, and stalk harvest on September 2nd.

Virus strains

CMV + PVY: from a plant found in 1998 to be infected by both CMV and PVY (according to DAS-ELISA tests), we collected leaf fragments which were conserved through dehydration on $CaCl_2$ (Bos technique). Later, these fragments were hydrated and served as inoculum source for infesting an ITB30 plant in the greenhouse, which proved to be infested by both viruses (DAS-ELISA). This ITB30 plant served as the source of inoculum for the treatment referred as "CMV+PVY".

ELISA controls visual symptom notation

Control DAS-ELISA tests from field inoculated plants were made. Results were consistent with expectations, however, in some plants from the CMV+PVY treatment, only CMV gave a positive signal. Natural occurrence of viral infestation due to aphids was higher in the 1999 experiment. Visual notation of symptoms was performed on July 29th, and at this date, 22% of the non-inoculated Kentucky 17 and 1% of the non-inoculated ITB501 plants were showing necrotic symptoms.

Results

Results of the 1998 and 1999 experiments are shown in table 4 and 5, respectively.

1998 experiment

In 1998, field heterogeneity were present in replication n° 1. Removal of data from this replication improved the quality of results; means and statistical results reported in table 4 for chemical traits refer only to replication n° 2, 3 and 4. Both yield and leaf quality are decreased by CMV or PVY infestation. Consistently with previous results, nitrate and nitrogen levels are higher in inoculated plants. Increases obtained with CMV are more important than those obtained with PVY. Total alkaloids contents seem not to be influenced by either type of viral infestation. Nornicotine contents are slightly higher in infested situations, but this slight difference is non-significant.
Leaves samples for smoking tests include replication n° 1. Despite this, the effect of virus infestation on specific activity is significant. The average increase, when compared to non-inoculated controls, is about 17%. CMV gives about the same increase as PVY. Every inoculation gave higher specific activity than the non-inoculated control, except one (Kentucky 17, CMV).

1999 experiment

Field heterogeneity was low, and quality of results was satisfactory. Every replication is involved in the data presented (table 5). The same trends as in 1998 can be seen, however the effect of viral infestation on chemical balance is more pronounced here. In particular, nitrate contents are substantially higher in inoculated treatments than in their non-inoculated counterparts. Again, the total alkaloid content does not seem to be influenced by either type of virus. For nornicotine, an increase linked to viral infestation (in particular CMV) is revealed in this trial.

The effect of virus infestation on specific activity is also more pronounced here than in the 1998 experiment. Specific activity from Kentucky 17 is increased up to 40% (CMV+PVY). If the healthy control had not been contaminated by naturally occurring viruses, one may expect that its specific activity would have been lower, then the contrast with inoculated plants would have been more important. With the variety ITB501, viral effect on specific activity is much lower (10%) than with Kentucky 17. This can be connected with the fact that ITB 501 has a genetic resistance factor to PVY. Accordingly, the specific activity of ITB501 seems more affected by CMV (4165) than by PVY (4010).

Discussion

Nitrate and non-nitrate N

By multiplying the nitrate contents by 14/62, one get an estimate of the N contained in nitrates. It can be deducted from table 5 that this fraction of the total N is explaining partly, but not entirely, the level of total N that is observed in PVY or CMV infested plants. The non-nitrate part of the total N is also higher in infested plants. However, the share of nitrate-N in the total-N is more important in virus infested plants (9,2 to 14,4%) than in their healthy controls (7,1 and 8,9%).

Sampling effect on smoke properties

Since the whole leaves have been shredded, including the nitrate-rich midrib, properties of

the smoke obtained may be partially due to nitrate from the midrib. The higher specific activity found for virus infested plants might then be due to the higher nitrate content of their midribs. This would suggest that differences, in specific activity, between virus infested and healthy plants, would have been less important with cigarettes made from strips. This has to be checked by running further experiments with a more adequate sampling.

Interaction between viral multiplication and plant growth

Plants have been inoculated 24 to 27 days after transplanting, and at this date, the essential part of the aerial growth was still to be completed. Due to their viral nature, CMV and PVY can only multiply in growing tissues (DAS-ELISA tests in mature leaves of infested plants are generally negative). Then, the alteration of the chemical balance of leaves is likely to be due to the result of interaction between viral multiplication and plant growth.

This interaction decreases the speed of growth, which can be attributed partly to a less efficient photosynthesis, linked with the mosaic aspect (19). The way by which chloroplasts are affected by virus multiplication is not clear. It has been shown that the activity of chloroplastic RNase from tobacco protoplasts infected with PVY is twice as high as in healthy protoplasts (20). Yield reduction, anyhow, is the final result.

Our results, as well as previous ones, suggest that viral interaction with growth also seems to affect the balance between non-N molecules (carbohydrates, organic acids) and N-containing molecules. Mechanisms underlying these alterations are not well known.

Many recent results show that viral genes interfere with cellular gene expression in plants. Furthermore, it has been shown that "viral movement protein can exert an effect on sugar metabolism and resource allocation at sites distant from their expression" (18). This latter finding suggests that, not only at the cellular level, but also at the whole organism level, viral genes may influence chemical balances. It is likely that it will be necessary to consider viral effects at the whole organism level for getting a better understanding of the results shown here.

Conclusion

CMV and PVY infestation modify the burley tobacco cured leaf chemical balance. They increase the specific activity of the smoke obtained from whole leaves, including midribs. It will be of interest to assess the consequence of such virus infestations when considering cigarettes made from strips (excluding midribs).

Cured leaves from infested plants show a substantially higher nitrate content than cured leaves from healthy plants. The non-nitrate part of the total nitrogen content is also higher. In the experiment where the viral effect was the more pronounced, a higher nornicotine content has been observed.

These effects are connected to the intensity of mosaic or necrotic symptoms. Tobacco varieties showing resistance, based on symptom expression, are also more stable in their chemical composition when challenged with these viruses, and their smoke specific activity is less influenced.

Mixed infections with both viruses create heavier symptoms, more important chemical

changes and more pronounced specific activity increases.

These results are consistent with alterations of the chemical balance observed with several other single stranded RNA+ virus affecting tobacco (TMV, TEV, TRSV, TVMV). It would be of interest to check whether these other viruses also affect the smoke specific activity in the same way as CMV and PVY seem to do.

Tables 1-5

Table 1. Methods used in 10 studies of viral effect on tobacco leaf chemistry (referring to data shown in table 2)

N°ref	Authors	Variety	Virus	Trial year	Trial type	Inoculation technique
3	HOLDEN and TRACEY, 1948	White Burley	TMV	1946	Glasshouse	mechanic (rubbing)
4	WOLF and WOLF, 1955	Dixie Bright 101	TRSV		Field grown tobacco.	natural infestation. Paired of leaves from diseased/h plants from the same stal position in the same field.
5	SIEVERT, 1978 a	BURLEY 49	PVY	1971, 1972	Field trial	mechanic (silicon carbide
6	SIEVERT, 1978 b	BURLEY 49	PVY	1972	Field trial	mechanic (silicon carbide
7	SIEVERT, 1978 c	BURLEY 37	PVY, TMV+PVY, TMV	1968, 1969	Field trial, randomised blocks	mechanic (silicon carbide
8	SIEVERT, 1978 d	11 Burley varieties	PVY	1971, 1972	Field trial, split-plot	mechanic (silicon carbide
9	PIRONE and DAVIS, 1977	BURLEY 37, BURLEY 21, KENTUCKY 10	TVMV		Field trial	mechanic
10	DIALLO and MULCHI, 1981	Md 609	TMV, TEV, TMV+TEV	1977, 1978	Field trial, split-plot	mechanic (carborundum)
11	LATORRE and FLORES, 1984	NC 744, COKER 86	PVY		Field trial	mechanic (carborundum)
12	PIRO, PICCIRILLO, and AVIGLIANO, 1992	KENTUCKY 17	PVY, CMV, PVY+CMV	1989	Field trial	mechanic (Carborundum)

*** days after transplanting**

Table 2. Leaf chemical traits from virus infested (I) and healthy controls (H) in 10 studies

Authors	Variety	Virus	total nitrogen		
			H	I	%I/H*
HOLDEN and TRACEY, 1948		TMV	3,19	4,19	131
WOLF and WOLF, 1955	Dixie Bright 101	TRSV	1,80	2,49	138
SIEVERT, 1978 a	BURLEY 49	PVY	3,45	4,10	119
SIEVERT, 1978 b	BURLEY 49	PVY	3,38	4,27	126
SIEVERT, 1978 c	BURLEY 37	PVY	4,40	4,42	100

SIEVERT, 1978 c	BURLEY 37	TMV+PVY	4,40	4,51	103
SIEVERT, 1978 c	BURLEY 37	TMV	4,40	4,43	101
SIEVERT, 1978 d	BURLEY 21	PVY	3,81	4,74	124
SIEVERT, 1978 d	BURLEY 49	PVY	4,27	4,83	113
SIEVERT, 1978 d	BURLEY 37 × L8	PVY	3,86	4,65	120
PIRONE and DAVIS, 1977	BURLEY 37	TVMV	5,00	5,53	111
PIRONE and DAVIS, 1977	KENTUCKY 10 **	TVMV	4,71	4,75	101
DIALLO and MULCHI, 1981	Md 609	TMV	3,66	3,88	106
DIALLO and MULCHI, 1981	Md 609	TEV	3,66	3,86	105
DIALLO and MULCHI, 1981	Md 609	TMV+TEV	3,66	4,05	111
LATORRE and FLORES, 1984	NC 744 **	PVY	1,26	1,75	139
LATORRE and FLORES, 1984	COKER 86	PVY	1,80	2,63	146
PIRO et al., 1992	KENTUCKY 17	PVY	5,91	5,83	99
PIRO et al., 1992	KENTUCKY 17	CMV	5,91	6,03	102
PIRO et al., 1992	KENTUCKY 17	PVY+CMV	5,91	6,27	106
Mean of results by virus	**number of results**				
	3	TMV	3,75	4,17	111
	1	TRSV	1,80	2,49	138
	2	TVMV	4,86	5,14	106
	1	TEV	3,66	3,86	105
	9	PVY	3,57	4,14	116
	1	CMV	5,91	6,03	102

* %I/H = value obtained from infested plants, expressed in % of the healthy control.
** varieties showing some degree of tolerance / resistance to the inoculated virus.

Table 3. Field notation scales for virus symptoms

CMV
0 : healthy plant
1 : slight mosaic on a few leaves (-20%)
3 : mosaic on 20 to 40 % of leaves
5 : mosaic on 40 to 60 % of leaves
7 : mosaic on 60 to 100% of leaves and slight distortion.
9 : mosaic on all leaves with important distortion and narrow leaves.

PVY
0 : healthy plant
1 : a few mosaics
3 : mosaics
5 : important mosaics
7 : 1 to 2 leaves with necrotic veins
9 : 3 to 5 leaves with necrotic veins
11 : necrotic stalk

Table 4. 1998 results of non-inoculated versus PVY or CMV inoculated Burley tobacco varieties

Inoculation	Variety	Tot. nitrogen		Nitrate		Total alkaloids		Nornicotine test		Yield
		% d.m.	nk	% d.m.	nk	% d.m.	nk	% d.m.	nk	kg/ha
None	Kentucky 17	4,0		1,9		4,1		0,28		3082
	ITB 2204	3,1		1,3		3,1		0,23		4442
	TN 86	3,5		2,1		3,3		0,45		4205
	ITB 218	3,9		2,3		3,8		0,32		3480
PVY	Kentucky 17	4,5		2,6		4,2		0,30		2086
	ITB 2204	3,7		2,3		2,8		0,20		4280
	TN 86	3,3		2,0		2,8		0,50		3967
	ITB 218	4,1		2,6		3,9		0,42		3666
CMV	Kentucky 17	4,9		2,8		4,2		0,32		2516
	ITB 2204	4,2		3,6		3,0		0,19		3649
	TN 86	3,9		2,7		3,1		0,48		3635
	ITB 218	4,4		3,1		4,6		0,32		3400
Means										
None		3,6	c	1,9	b	3,6		0,32		3802
PVY		3,9	b	2,4	b	3,4		0,35		3500
CMV		4,4	a	3,0	a	3,8		0,33		3300
Kentucky 17		4,5	a	2,4		4,2	a	0,30	b	2561
ITB 2204		3,7	c	2,4		2,9	b	0,21	c	4124
TN 86		3,6	c	2,2		3,1	b	0,48	a	3936
ITB 218		4,1	b	2,6		4,1	a	0,35	b	3515
bloc		ns		ns		ns		ns		ns
inoculation		*		*		ns		ns		*
variety		*		ns		*		*		*
variety × inoculation		ns		ns		ns		ns		*

* significant at the 5% level
nk = Newman-Keuls groups

Table 5. 1999 results of non-inoculated versus PVY, CMV, or CMV+PVY inoculated Burley tobacco varieties

Inoculation	Variety	Total nitrogen		Nitrate		Total alkaloids		Nornicotine test		Y
		% d.m.	nk	% d.m.	nk	% d.m.	nk	% d.m.	nk	kg/h
None	Kentucky 17	3,8	cd	1,5	c	2,99	ab	0,26	c	247
	ITB 501	3,5	d	1,1	c	3,36	ab	0,32	bc	304

PVY	Kentucky 17	4,9	b	3,0	ab	3,48	ab	0,30	bc	1818
	ITB 501	3,4	d	1,0	c	2,85	b	0,40	abc	2875
CMV	Kentucky 17	4,6	b	2,8	ab	3,49	ab	0,44	ab	1816
	ITB 501	3,9	c	1,6	c	3,48	ab	0,38	abc	2414
PVY+CMV	Kentucky 17	5,2	a	3,3	a	3,52	a	0,50	a	1479
	ITB 501	4,1	c	2,3	b	3,38	ab	0,35	bc	2603
Means										
None		3,7	c	1,3	c	3,18		0,29	b	2757
PVY		4,2	b	2,0	b	3,17		0,35	ab	2346
CMV		4,3	b	2,2	b	3,48		0,41	a	2115
PVY+CMV		4,7	a	2,8	a	3,45		0,43	a	2041
Kentucky 17		4,6	a	2,7	a	3,37		0,38		1896
ITB 501		3,8	b	1,5	b	3,26		0,36		2734
bloc		*		*		ns		ns		ns
inoculation		*		*		ns		*		*
variety		*		*		ns		ns		*
variety × inoculation		*		*		*		*		*

* significant at the 5% level
nk = Newman-Keuls groups

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