P 4 - Verrier - Impact of PVY^N infection on burley cigarette smoke condensate properties: an assessment of the protection conferred by the «va» gene

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Impact of PVY^N infection on burley cigarette smoke condensate properties: an assessment of the protection conferred by the «va» gene.

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Summary

It has been formerly shown that PVY^N infection of Burley tobacco was associated with higher values for total nitrogen, nitrate, and cigarette smoke condensate (CSC) mutagenic activity (Ames test, S9 activation, *S. typhimurium* strain TA98), when compared to non infected counterparts.

This previous work was based on cigarettes made from whole leaves, comprising the midrib, a part of the leaf that contains high levels of nitrate. It was necessary to check to which extent PVY^N infection had the same effect when using cigarettes made from strips, therefore excluding the midrib. It was also important to assess to which extent the *va* gene, which confers partial resistance to PVY^N, contributes to diminish the effect of PVY^N infection on CSC mutagenic activity.

Two couples of near isogenic lines, one member of each bearing the [*va va*] genotype, the other one bearing [*VaVa*], were grown according to a split-plot design with 3 replicates. Main plots were ascribed to couples, sub-plots to virus challenge (PVY^N inoculation / no inoculation), and sub-sub-plots to genotypes. Chemical analyses were performed on cured leaves from each individual plot. In order to get sufficient samples for threshing, replicates were gathered within each combination of treatments, threshed, fine cut and manufactured to obtain cigarettes.

Using a linear smoking machine, two runs of mechanical ISO smoking were implemented for each of the 8 cigarette samples. The obtained CSCs were assessed for mutagenic activity, in two independent series of Ames tests (S9 activation, TA98 *S. typhimurium* strain).

Expressed as a number of revertant colonies per mg of CSC, the mutagenic activity was significantly increased by PVY inoculation. A 21% increase was observed for PVY susceptible [*Va Va*] tobaccos, whereas a lower, but still significant increase (10%) was observed for PVY^N resistant, [*va va*] counterparts.

These results confirm that cigarettes from strips of infected tobacco leaves are affected by in-field PVY^N infection, leading to a higher CSC mutagenicity. They also suggest that the *va* resistance contributes to diminish this effect, despite not entirely suppressing it.

Introduction

In as early as 1930, Dunlap reported that the C/N balance was modified by viral infections in several crops. In "yellow diseases" this ratio was increased, whereas in "mosaic diseases", it was decreased. The latter case included tobacco as one of the main examples.

The effect of "mosaic" in tobacco was studied in further works, where the viral agent could be better characterised. However, only few references have been devoted to studying this effect on chemical balance of cured leaves from field grown tobacco.

Tables 1 and 2 group results of such studies. Unrelated viruses from different genera, like PVY, CMV and TMV, have the same global impact on the cured leaf C/N balance, resulting in higher total nitrogen and nitrate contents. The non-nitric part of the total nitrogen is also increased. This suggests a general trend associated with "mosaic", since all these viruses are causing this type of symptom.

In contrast with this trend, the alkaloid content seems not consistently changed. In some cases no significant modification could be seen, and significant increases or decreases have been reported.

Nitrogen content has been shown to be positively related with mutagenic activity of cigarette smoke condensate (CSC), as measured using the Ames test with S9 mix activation and the TA98 strain of *Salmonella typhimurium* (Mizusaki, 1977). Since virus infections do influence this trait, it was of interest to check whether they would induce a higher CSC mutagenic activity.

Concerning CMV and PVY in burley tobacco, experiments showing that such an influence was significant have been reported (Verrier et al., 2001). These were based on cut-rag tobacco from whole leaves, comprising the midrib. Since chemical composition of the midrib is noticeably different from that of lamina, the exact impact of these infections on laminas, from which are made strips, which account for the most important part of the cigarette, was to be assessed.

Another point to be addressed was the effect of the *va* resistance to PVY^N. Compounds secreted by trichomes, which contribute to the smoke flavour, are in lower amount in (*va va*), resistant varieties. In turn, these varieties seem less prone to CSC mutagenic activity increase when infected by PVY^N. A more exact assessment of the various *va* effects is therefore of interest.

Materials and Methods.

Data presented here refer to a field trial grown in the location Bergerac in 2001, and involve two couples of burley lines challenged or not with PVY^N.

Plant materials.

The Burley lines PC1, PC2, and PC3 have been obtained from a backcrossing program (Figures 1 and 2) aiming at introducing the *va* gene from VAM (Koelle, 1961) into a Kentucky 17 (Collins et al., 1978) background.

Fixation of PC1, PC2 and PC3 for the *va* or *Va* allele has been verified using artificial inoculation of PVY^N in greenhouse. Due to the tight linkage between *va* (allele from VAM origin) and a genetic condition preventing glandular trichomes to secrete exudates (Yamamoto, 1992), PC1 and PC3 are non-secretors, whereas PC2 and Kentucky 17 possess secreting trichomes.

PC1 and PC2 were considered to be respectively the (*va va*) and (*Va Va*) members of the near isogenic couple n° 1, PC3 and Kentucky 17 the (*va va*) and (*Va Va*) terms of couple n° 2.

Experimental design.

A split-plot design with three replications was used. Couples were considered as being main plots. Sub-plots were assigned to virus treatments (non-inoculated / PVY^N inoculated). Sub-sub plots in each sub-plot were devoted to (*va va*) and (*Va Va*) genotypes. The elementary plot consisted of one row of 27 plants. Distances were 90 cm between rows, and 39 cm between plants within a row.

Cultural practices.

Seeds of each line were germinated in 7-cm diameter pots containing a commercial potting mix. Two weeks after seeding, plants were manually transferred to multi-celled trays installed in a greenhouse for transplant production with the floating bed system. Insecticide treatments for aphid control were applied. Generally, viral contamination of tobacco by aphids does not occur in greenhouses, but starts in field after transplanting (Brachet et al., 2001). Transplanting was performed with a mechanical transplanter on May 28^{th}, 2001 in the same field as other burley trials. At this date all transplants were free from virus symptoms.

Production practices were those recommended for commercial burley tobacco. Insecticide treatments for aphid control were applied 15, 35 and 49 days after transplantation.

Virus strains, field inoculation and evaluation.

The PVY^N strain 94-129 from the pathotype 2 (Blancard et al., 1995) was inoculated to a set of resistant and susceptible varieties in a temperature controlled greenhouse (lower than 26 °C). This allowed checking that it was producing necrotic symptoms only on susceptible, (*Va Va*) varieties.

From this experiment, infected leaves of a susceptible cultivar were crushed in a mortar. For 1 g of leaf, 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 introduced into a heat-insulated container kept at low temperature (4 °C) and brought immediately in field for inoculation. Inoculation was performed 16 days after transplanting. Two leaves /plant were gently rubbed with the solution.

Respectively 29, 40 and 58 days after inoculation, visual expression of symptoms was scored according to the scales shown in Table 3, on a plant by plant basis.

Harvesting, curing and sampling.

Stalk-cut harvest was done 92 days after transplanting. End of row plants were not harvested.

Air-curing took place in a plastic tunnel with control of excessive moisture using an air heating device and opening frames. At the end of cure, leaves were removed and grouped into 4 stalk positions. Each stalk position was weighed and scored for quality on a scale from 1 (poor) to 5 (best). An index was computed by combining these scores with the respective weights.

In each elementary plot, a representative sample of whole leaves, comprising the midrib, from the third (upper-middle) and fourth (tips) stalk positions grouped together, was ground to 500 µ powder for chemical determinations.

In each of the 8 combinations of treatments, the three replicates were gathered (leaves from third and fourth positions) in order to get enough material for threshing, fine cutting and making cigarettes. A representative sample of the obtained strips was ground to 500-µm powder to get chemical assessments. The rest of the strips was fine cut (0,8 mm).

Cigarette making and smoking test.

Cigarettes without filter (diameter: 7,9 mm, length: 70 mm) were machine made. For each batch, the machine was set in order to get a draw resistance of about 70 mm (water column). Cigarettes that weighed more than 60 mg apart from the average weight for their batch were discarded. Remaining cigarettes that were under 30 or above 100 mm draw resistance were discarded.

Due to the limited amount of material in some batches, the overall goal of a 70 mm draught resistance could not always be reached. Six of the eight batches had a mean resistance between 60 and 80 mm, however one was at 48 and one at 84 mm (Table 4).

A part of these cigarettes was submitted to the ISO3308 smoking test on a rotative machine, from which Nicotine Free Dry Particulate Matter (tar) and Nicotine yields per cigarette have been measured.

Another part was smoked in order to get cigarette smoke condensate (CSC). The eight batches were submitted to two runs of the smoking linear machine, yielding 16 CSC samples. These were diluted into DMSO (10 mg/ml) and, immediately after extraction, stored at –80 °C.

Ames tests.

Two independent runs of the mutagenicity test described by Ames (Ames, 1973) using S9 metabolic activation and the TA98 strain of *Salmonella typhimurium* were performed. In each run, 6 doses of each CSC sample were studied: 0.05, 0.1, 0.20, 0.25, and 0.30 mg / plate. Numbers of revertant colonies were counted, and the slope of the linear regression of these numbers on doses was computed. This slope is expressed in number of revertant colonies for 0,175 mg of CSC, and is referred to hereafter as CSC mutagenic activity.

Chemical determinations.

Dry matter has been determined from oven dehydration. Nitrate content was obtained through CORESTA method n° 36 (Continuous Flow Analysis). Near Infrared Reflectance Spectrometry (NIRS) was used to estimate total alkaloid content, total nitrogen, ashes, and

ammonia. Calibration equations for this method have been developed internally (Poisson et al., 2004).

Nicotine, myosmine, anatabine, anabasine and nornicotine contents were measured by High Performance Liquid Chromatography (internal method).

Statistical treatments.

The multifactor analysis of variance module of the software STATGRAPHICS Plus version 5 was used to test the statistical significance of the different effects, and to produce Figures 4 - 6.

Results

Results of the experiment are shown in Table 4, Figure 4 (whole leaves), 5 (strips and cigarettes) and 6 (Ames test).

Expression of viral symptoms.

Plant growth was homogeneous throughout the trial. Viral symptoms appeared 3 weeks after inoculation, and at this date they were restricted to susceptible and inoculated plants. Later on, evidence of natural virus contamination by aphids could be deduced from new symptoms seen on non inoculated plants, despite insecticide treatments. This contamination was mostly due to CMV and affected principally the replication n° 3 in the field. Means of symptoms notations, and frequencies of plants having a symptom score greater than 5, are shown in Figure 3 for each of the three reading dates, for susceptible plants only.

Despite this natural contamination by CMV, there was still a strong difference between non inoculated and inoculated plots, linked to the effect of PVY^N in inoculated plots.

Difference between resistant and susceptible lines appeared always clearly in PVY^N inoculated plots, resulting in a highly significant Genotype × Virus treatment interaction (Figure 4) for the average symptom note.

Leaf yield and quality.

Both leaf yield and quality where affected in a rather similar way by PVY^N inoculations. These traits were only slightly modified in resistant lines, whereas they were clearly depressed in their susceptible counterparts. Resistant lines challenged with PVY showed a slight reduction in leaf quality scoring but remained nearly constant as regards yield. Couple n° 1 gave a higher yield than couple n° 2.

Leaf chemical composition.

Chemical compounds shown in Table 4 all contain nitrogen. For these traits, contents in strips tend to be higher than in whole leaves, which can be attributed to the removal of the midrib, low in nitrogen. Figures 4 and 5 show a general tendency for higher contents in leaves from PVY^N inoculated plants, when compared to non-inoculated counterparts. This difference is more pronounced for total nitrogen and nitrates than for alkaloids and ammonia.

Moreover, nitrogen and nitrates display clearly a Genotype x Virus treatment interaction: resistant lines show only slightly higher levels in PVY^N infested than in non inoculated plots, whereas susceptible counterparts show much higher levels. As a consequence, interaction plots (Figures 4 and 5) have the same pattern as the one for visual symptom expression.

This cannot be seen with ammonia and alkaloids, for which there are little differences between resistant and susceptible lines, in either situation.

Total nitrogen content was slightly but significantly higher in couple n° 1, with a difference from couple n° 2 of about 0,2 % of the dry matter. It seems this has to be attributed mostly to nitrate and ammonia, for couple n° 1 was also higher than couple n° 2 in both these traits.

There seemed to be a general tendency towards slightly higher ammonia, total alkaloids, nicotine, anatabine and nornicotine contents in (*va va*) lines, however in all cases but one (ammonia in whole leaves, P (F) = 0,05) the difference was non-significant.

Myosmine could not be detected in any of the samples, and anabasine was at a very low level (0,01 to 0,02 % of the dry matter) in all treatments.

The ratio of nornicotine to total alkaloids was below 6% in any case, indicating that the burley lines studied here are non converters, and that in such lines this ratio does not seem to be substantially modified by PVY^N inoculation. It can be seen, however, that PC3 ("vava" member of couple n° 2) had higher ratios than other lines.

Physical characters of cigarettes and smoking test results.

The average cigarette weights of the 8 batches ranged between 764 and 921 mg. Both batches from the resistant lines, non-inoculated, had the lowest weights, which is clearly shown in the corresponding plot in Figure 5. Despite being clear in the diagram, lack of replication resulted in non-significant differences. Draw resistance and tar yield of these two batches were also lower than those of the other six batches, which resulted in diagrams similar to the one of cigarette weights.

Both couples showed close levels for physical characters of the cigarettes as well as for smoking test results.

NIRS estimates for petroleum ether extracts (PE) (data not shown), which contain trichome secretions, had a pattern which can be related to the one found for cigarette weights.

Susceptible (*Va Va*) lines possess secreting trichomes, whereas resistant (*va va*) lines do not. However, PE estimates are similar for all lines in PVY^N infested situations, and only the non-inoculated situation reveals clearly higher estimates for (*Va Va*) lines. This suggests that trichome secretions would be to some extent diminished upon PVY^N infection in secretor lines. In non-inoculated situations, secreting trichomes of the (*Va Va*) lines operate, whereas non-secreting (*va va*) lines still have no secretion, hence the difference for PE.

Since trichome secretions have a sticky character, it can be hypothesised that they interfered with the process of cigarette making, resulting in higher weights and slightly higher draw resistance and tar for cigarettes from susceptible lines in non inoculated situation.

Nicotine yields per cigarette were substantially higher (+ 20%) with cigarettes from PVY^N

infected tobacco, whereas contents in strips were only slightly higher. This led to slightly but consistently higher nicotine to total condensate matter ratios in CSCs from PVY^N infected tobacco (15.4 - 16.5 %, to be compared to 14.0 - 14.7 % in healthy tobacco). This effect can only be partially related to the slightly higher nicotine content, cigarette weights and tar yields in tobacco from PVY^N infected plants. A higher nicotine transfer rate in tobacco from PVY^N infected plants may be suspected.

Ames test.

Data have been interpreted by multifactorial variance analysis, first according to the following model:

Model 1:

Revertant count = Constant term + CSC Dose effect + Term effect + Ames test run effect + Smoking run effect + Two order interactions + Residual.

The word "term" in this model refers to the 8 combinations of virus treatments, couples and genotypes.

Results are given in Table 5 and Figure 6. All the main categories had significant effects. Estimates of the effects of the 8 terms are shown in Figure 6.

The Dose × Term interaction reflects the fact that, at high doses, differences between terms were greater than at lower doses, as shown by the "interaction plot" of Figure 6. Similarly, differences were larger in one smoking run than in the other one, and in one Ames test run than in the second one (interactions BD and BC, top of Table 5). The ranking of the different terms, however, was conserved in every smoking run and replicate of the Ames test.

Introducing genotype, couple and virus treatment instead of "term" led to the following model:

Model 2:

Revertant count = Constant term + CSC Dose effect + Virus treatment effect + Genotype effect + Couple effect + Ames test run effect + Smoking run effect + two order interactions + Residual.

Results are given in Table 5. Two order interactions that had been excluded were non-significant.

Virus treatments and genotype effects were significant, whereas couples were globally equivalent.

PVY^N inoculation resulted in higher revertant counts. Resistant lines had lower revertant counts than susceptible lines, but the difference was significant only in PVY^N inoculated situation.

Similarly to model 1, Dose × Virus treatment and Dose x Genotype interactions reflect the fact that, at high doses, differences between these categories were greater than at low doses. The interaction plot of Doses x Ames test run (Figure 6) also can be explained in this way.

Couple × Smoking run interaction is not of the same nature, since in this case the relative ranking of couples is not conserved. However, confidence intervals are nearly overlapping, which shows that this interaction, despite significant, does not involve big differences. Couple n° 1 had slightly more total N and ammonia than couple n° 2, therefore should have yielded a few more revertants, which happened in smoking run 1 only, hence the interaction.

The Virus treatment × Genotype interaction reflects the fact that a difference could be seen between resistant and susceptible lines when inoculated with PVY^N, whereas there was no clear difference in non inoculated situation. The pattern of the corresponding interaction plot (Figure 6) is very similar to the one already encountered with total nitrogen, nitrates and viral symptoms.

The Virus treatment × Couple interaction was also significant. It seems that couple 2 tended to have a lower revertant count than couple 1 in non-inoculated situation, but was more affected by PVY^N, resulting in higher revertant counts in infected situation.

Among the 8 studied terms, a linear relation can be seen between total nitrogen in strips and mean revertant counts (r^2= 0,80). At 0,175 mg of CSC, the global slope of this relationship is close to 100 revertants for 1 % of total nitrogen.

In more details, it seems that couple 2 had a greater slope than couple 1.

The relationship between mean revertant counts and total nitrogen in strips is the tightest that can be found in these data. Links with nicotine yields in cigarettes or nitrate content in whole leaves are weaker (r^2 = 0,59 for both traits).

Discussion

Effect of PVY^N inoculation.

The total amount of nitrogen harvested in leaves and still present in cured leaves can be estimated from the product of cured leaves yield by total nitrogen content. Results are slightly higher for PVY infested situations. This suggests that, despite a lower total yield, infected plants do uptake from the soil at least as much nitrogen as healthy plants. The much higher amounts of nitrates found in whole leaves from infected plants also supports this concept of increased nitrogen extraction. It seems likely that nitrate uptake is triggered by the viral infection.

Results presented here confirm that PVY^N infection of burley tobacco affects the chemical constitution of strips, leading to a higher CSC mutagenic activity. With the variety Kentucky 17, the increase, relatively to the non-inoculated control, is 31%. This variety was also tested in former trials (Verrier et al., 2001), and working with whole leaves the observed increases were 12 % and 27 %, depending on the trial. Then, it can be estimated that this PVY^N induced effect is at least as important in strips as in whole leaves.

PVY^N infected tobaccos undoubtedly contain proteins and other compounds that are absent from healthy ones. Most of the plant viruses are single stranded RNAs that are able to redirect the cellular machinery towards their own translation into viral proteins, absent from

non-infected plants (see Hull, 2002 for review). In the particular case of PVY, some of these proteins (NIa, NIb, CI etc.) accumulate inside cells and form characteristic inclusion bodies that can be seen through microscopic examination. Viral particles themselves are primarily constituted from repeated molecules of the coat protein, which account for about 90% of their mass. Another, more indirect source of compounds specific to pathogen infection is provided by the plant defence mechanisms, resulting in the synthesis of various protein or non protein molecules.

Contribution of these specific compounds to the observed higher CSC mutagenic activity is however difficult to assess from these results.

The linear relationship between total nitrogen and CSC mutagenic activity found here is consistent with previous results (Mizusaki, 1977). In particular, the slope found here, about 100 revertants more for one percent more nitrogen, at 0,175 mg of CSC, seems lower than or equal to what was formerly reported. In Mizusaki study, an increase of 400 revertants for 1% of total nitrogen was found at 0,5 mg CSC. Assuming a linear dose - response relationship, this is equivalent to 140 revertants at 0,175 mg.

This lower or equal slope apparently suggests that nitrogenous compounds more specifically present in cured leaves from PVY^N infected tobacco are not qualitatively more mutagenic than those present in cured leaves from healthy tobacco. Only the relative increase of these nitrogenous compounds in the total dry mass would then explain the PVY^N induced differences.

It must be stated, however, that CSC mutagenic activity as revealed by the Ames test is not the simple addition of contributions from each individual compound, and rather is a result of antagonistic or synergistic effects. One of these effects (Lee et al., 1996) involves nicotine and other alkaloids, which inhibit the mutagenicity of some tobacco specific N-nitrosamines. The underlying mechanism is linked to cytochrome P450 activity, which in some cases seems to be repressed by nicotine. Then, it can be hypothesised that the consistently higher nicotine content of CSC form PVY^N infected tobacco has antagonistic effects against other compounds.

Then, it cannot be ruled out that PVY^N infection directly or indirectly would trigger the synthesis of nitrogenous compounds that have potentially a higher mutagenic effect than those found in healthy tobacco. This would be counteracted by higher antagonistic effects, at least due to the higher nicotine content, than in CSC from healthy tobacco. The final result would then be an increase of CSC mutagenicity proportional to the increase of the nitrogen, in percent of the dry matter of infected leaves, with the same general proportionality coefficient than in healthy tobacco.

Heterocyclic amines derived from the pyrolysis of amino acids and proteins have been shown to be the most potent mutagens in the Ames TA98+S9 system (Sugimura, 1985. Review in Massey, 2002), in particular non-IQ forms created by pyrolysis of glutamic acid and tryptophan. An interesting information, in order to better assess the above presented hypothesis, would then be provided by the amino acid composition of proteins present in PVY^N infected tobacco, in particular those that are virus related or produced by the host plant through defence mechanisms.

Effect of the va gene.

An effect of trichome secretions, which are diminished or suppressed in (*va va*) lines, on physical parameters of cigarettes has been suggested from results of this study, but remains to be confirmed.

All PVY^N affected traits are clearly more stable in (*va va*) lines than in their counterparts, when challenged with the virus. This comprises total nitrogen in strips as well as CSC mutagenic activity, and shows clearly the advantage of the *va* resistance. The protection conferred by *va*, however, is only partial.

Complementary resistance genes or cultural means of protection seem therefore necessary.

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Tables and Figures

Table 1. Methods used in 11 studies of viral effect on tobacco leaf chemistry (referring to data shown in Table 2)

Study **	Trial year	Trial type	Inoculation technique	D.I.*	Date of harvest	Cur
1	1946	Glasshouse	mechanic	20	30 - 35 days after inoc.	none - leav
2		Field grown tobacco.	natural infestation. Paired sets of leaves from diseased/healthy plants from the same stalk position in the same field.		at maturity	flue-c

3	1971, 1972	Field trial	mechanic	21	at maturity	air-c
4	1972	Field trial	mechanic	16	at maturity	air-c
5	1968, 1969	Field trial	mechanic	27 to 30	at maturity	air-c
6	1971, 1972	Field trial	mechanic	28 to 32	at maturity	air-c
7		Field trial	mechanic	21	at maturity	air-c
8	1977, 1978	Field trial	mechanic	28 and 42	at maturity	air-c
9		Field trial	mechanic	28	at maturity	flue-c
10	1989	Field trial	mechanic		at maturity	air-c
11	1999	Field trial	mechanic	27	at maturity	air-c
12	1968,1969	Field trial	mechanic	0	at maturity	Flue-c

* DI : number of days between transplanting and inoculation

** 1: Holden & Tracey, 1948 2: Wolf & Wolf, 1955 3: Sievert, 1978a 4: Sievert, 1978b 5: Sievert, 1978c 6: Sievert, 1978d 7: Pirone & Davis, 1977 8: Diallo & Mulchi, 1981 9: Latorre & Flores, 1984 10 : Piro & al., 1992 11: Verrier et al., 2001 12: Harman et al, 1971

Table 2. Leaf chemical traits measured from virus infested (I) and healthy controls (H) in 12 studies.

Study *	Variety	Virus	chemical traits (% d. m.) *					
			total nitrogen			nitrate		
			H	I	%I/H	H	I	%I/H
1		TMV	3.19	4.19	131			
2	Dixie Bright 101	TRSV	1.80	2.49	138			
3	Burley 49	PVY	3.45	4.10	119	0.43	0.52	121
4	Burley 49	PVY	3.38	4.27	126	0.12	0.17	142
5	Burley 37	PVY	4.40	4.42	100	0.17	0.25	147
	Burley 37	TMV+PVY	4.40	4.51	103	0.17	0.33	194
	Burley 37	TMV	4.40	4.43	101	0.17	0.22	129
6	Burley 21	PVY	3.81	4.74	124	0.51	0.70	137
	Burley 49	PVY	4.27	4.83	113	0.52	0.71	137
	Burley 37 x L8	PVY	3.86	4.65	120	0.42	0.42	100
7	Burley 37	TVMV	5.00	5.53	111	0.63	0.76	121
	Kentucky 10 ***	TVMV	4.71	4.75	101	0.56	0.61	109
8	Md 609	TMV	3.66	3.88	106			
	Md 609	TEV	3.66	3.86	105			
	Md 609	TMV+TEV	3.66	4.05	111			
9	NC 744 ***	PVY	1.26	1.75	139			
	COKER 86	PVY	1.80	2.63	146			
10	Kentucky 17	PVY	5.91	5.83	99			
	Kentucky 17	CMV	5.91	6.03	102			
	Kentucky 17	PVY+CMV	5.91	6.27	106			
11	Kentucky 17	PVY	3.77	4.90	130	1.50	2.97	198
	ITB501 ***	PVY	3.54	3.43	97	1.08	1.01	94

	Kentucky 17	CMV	3.77	4.61	122	1.50	2.78	185
	ITB501	CMV	3.54	3.93	111	1.08	1.61	149
	Kentucky 17	PVY+CMV	3.77	5.22	138	1.50	3.33	222
	ITB501	PVY+CMV	3.54	4.11	116	1.08	2.34	217
12	Hicks	TMV	2.41	3.11	129			
	NC 2512	TMV	2.26	2.96	131			
	NC 2326	TMV	2.38	2.99	126			
	SpG 36	TMV	2.49	3.00	120			
	Mc 30	TMV	2.43	3.15	130			
	# results							
Mean by virus	8	TMV	2.90	3.46	122	0.17	0.22	129
	2	TVMV	4.86	5.14	106	0.60	0.69	115
	11	PVY	3.59	4.14	119	0.59	0.84	134
	3	CMV	4.41	4.86	112	1.29	2.20	167

* 1: Holden & Tracey, 1948 2: Wolf & Wolf, 1955 3: Sievert, 1978a 4: Sievert, 1978b
5: Sievert, 1978c 6: Sievert, 1978d 7: Pirone & Davis, 1977 8: Diallo & Mulchi, 1981
9: Latorre & Flores, 1984 10 : Piro & al., 1992 11: Verrier et al., 2001 12: Harman et al., 1971.
** H : Healthy I : Infested %I/H = value from infested plants, in % of the healthy control.
*** varieties showing some degree of tolerance / resistance to the inoculated virus.

Table 3. Field notation scale for PVY and CMV symptoms.

PVY	CMV
0 : healthy plant	0 : healthy plant
1 : a few mosaics	1 : slight mosaic on a few leaves (-20%)
3 : mosaics	3 : mosaic on 20 to 40 % of leaves
5 : important mosaics	5 : mosaic on 40 to 60 % of leaves
7 : 1 to 2 leaves with necrotic veins	7 : mosaic on 60 to 100% of leaves and slight distortion.
9 : 3 to 5 leaves with necrotic veins	9 : mosaic on all leaves, important distortion and narrow leaves.
11 : necrotic stalk	

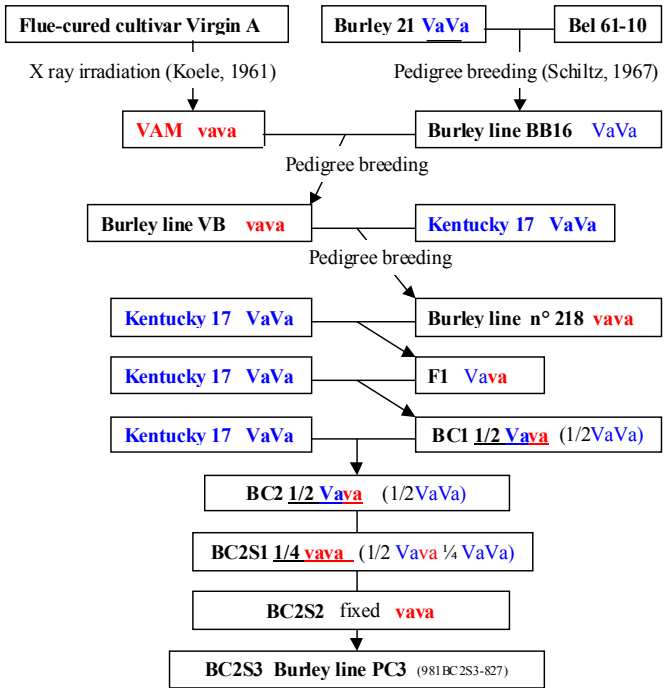


Figure 1. Origine of the Burley line PC3.

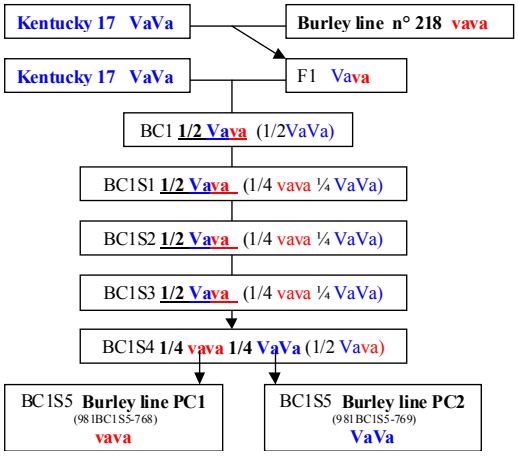
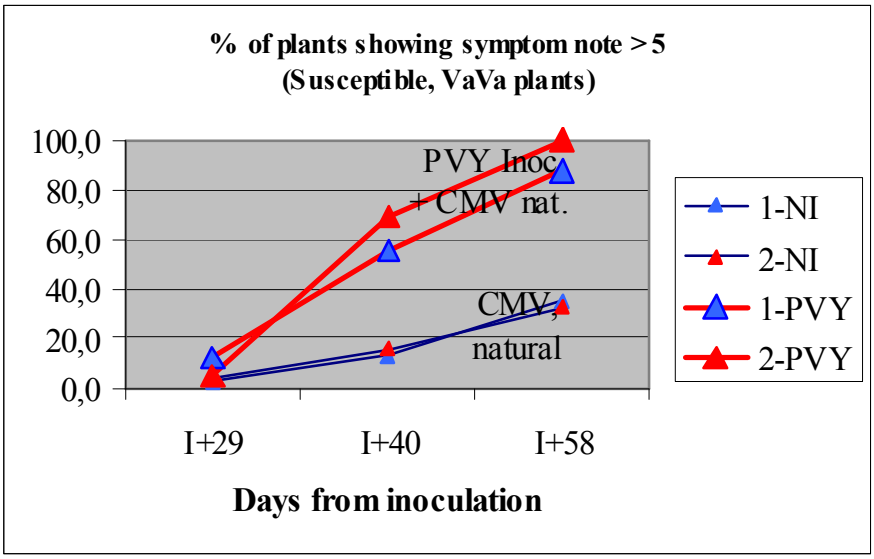


Figure 2. Origin of burley lines PC1 and PC2.



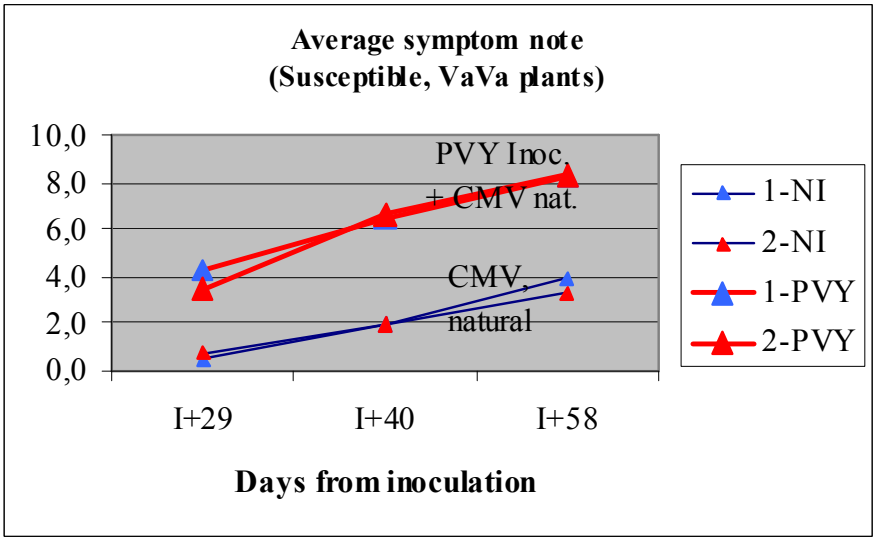


Figure 3. Frequency of plants showing necrotic symptoms, and mean symptom notation, for susceptible (VaVa) plants

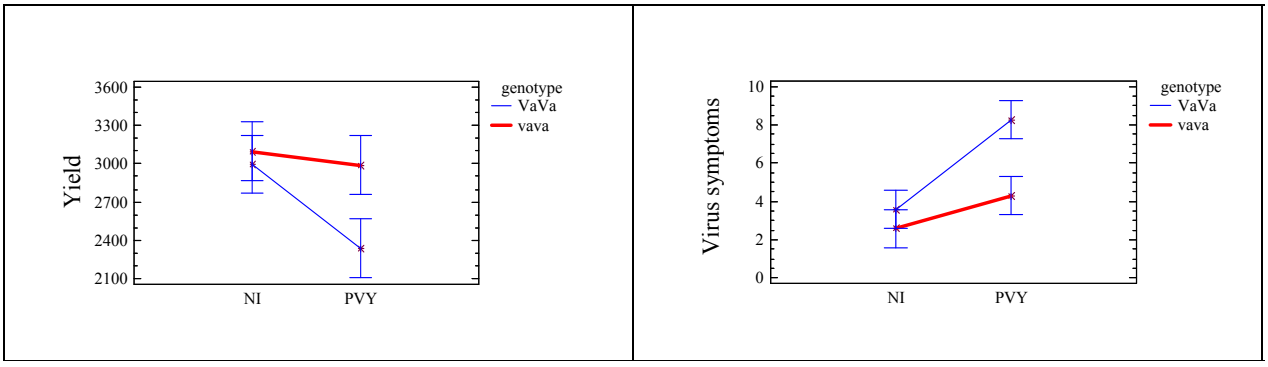
NI : non inoculated, PVY : PVYN inoculated, 1 : susceptible member of couple n° 1, 2 : susceptible member of couple n° 2

Table 4. Comparison of resistant (vava) and susceptible (VaVa) members of two couples of burley lines inoculated with PVY^N or not

Couple	Virus ttmt.	Geno-type	Field obs. & quality			Whole leaves				Strips after thresh			
						NIRS			Flow	NIRS			HI
			Yield	Virus Sympt.	Leaf quality	A	N	NH3	NO3	N	NH3	nic	anat
			kg/ha	(1-5)	(1-11)	% dm				% dm			% dm

1	NI	vava	3271	2,8	2,2	2,7	3,9	0,44	1,2	4,4	0,56	4,0	0,20
1	NI	VaVa	3320	3,9	2,4	2,8	3,6	0,41	0,7	4,3	0,57	4,3	0,20
1	PVY	vava	3191	4,1	2,0	3,1	4,6	0,53	1,7	5,0	0,58	4,6	0,23
1	PVY	VaVa	2347	8,3	1,1	2,7	4,8	0,48	2,1	5,5	0,55	4,5	0,23
2	NI	vava	2919	2,3	2,0	3,1	3,8	0,42	0,5	4,2	0,48	4,7	0,23
2	NI	VaVa	2667	3,3	1,8	2,6	3,5	0,39	0,4	4,2	0,50	4,1	0,19
2	PVY	vava	2782	4,5	1,7	2,9	4,0	0,45	0,6	4,6	0,52	4,6	0,23
2	PVY	VaVa	2335	8,3	1,5	2,8	4,6	0,45	1,8	5,3	0,51	4,6	0,23
Virus treatment means													
	Non inoculated		3044	3,1	2,1	2,8	3,7	0,42	0,7	4,3	0,53	4,3	0,21
	PVY^N		2664	6,3	1,6	2,9	4,5	0,48	1,6	5,1	0,54	4,6	0,23
Couple means													
	1		3032	4,8	1,9	2,8	4,2	0,47	1,4	4,8	0,56	4,3	0,21
	2		2676	4,6	1,7	2,9	4,0	0,43	0,8	4,6	0,50	4,5	0,22
Virus effect			s	s	s	-	s	s	s	s	-	-	-
Genotype effect			s	s	-	-	-	0,05	-	0,01	-	-	-
Couple effect			s	-	-	-	0,01	0,01	s	0,02	s	-	-
Virus x Gen. interact.			0,02	s	-	-	s	-	s	s	-	-	-
Means when non inoculated													
	va va / NI		3095	2,6	2,1	2,9	3,8	0,43	0,9	4,3	0,52	4,3	0,21
	VaVa / NI		2993	3,6	2,1	2,7	3,6	0,40	0,6	4,3	0,54	4,2	0,20
	Gen. effect / NI		-	-	-	-	0,01	-	0,02				
Means when PVY inoculated.													
	va va / PVY		2987	4,3	1,9	3,0	4,3	0,49	1,2	4,8	0,55	4,6	0,23
	VaVa / PVY		2341	8,3	1,3	2,7	4,7	0,47	1,9	5,4	0,53	4,6	0,23
	Gen. effect / PVY		s	s	0,02	-	0,02	-	0,02				

A : total alkaloids. N : total nitrogen, nic : nicotine, anat : anatabine, nor : nornicotine, ratio : nor
Wcig: cigarette mean weight DR : mean draw resistance, CSC mut. act. : revertant number estimate f



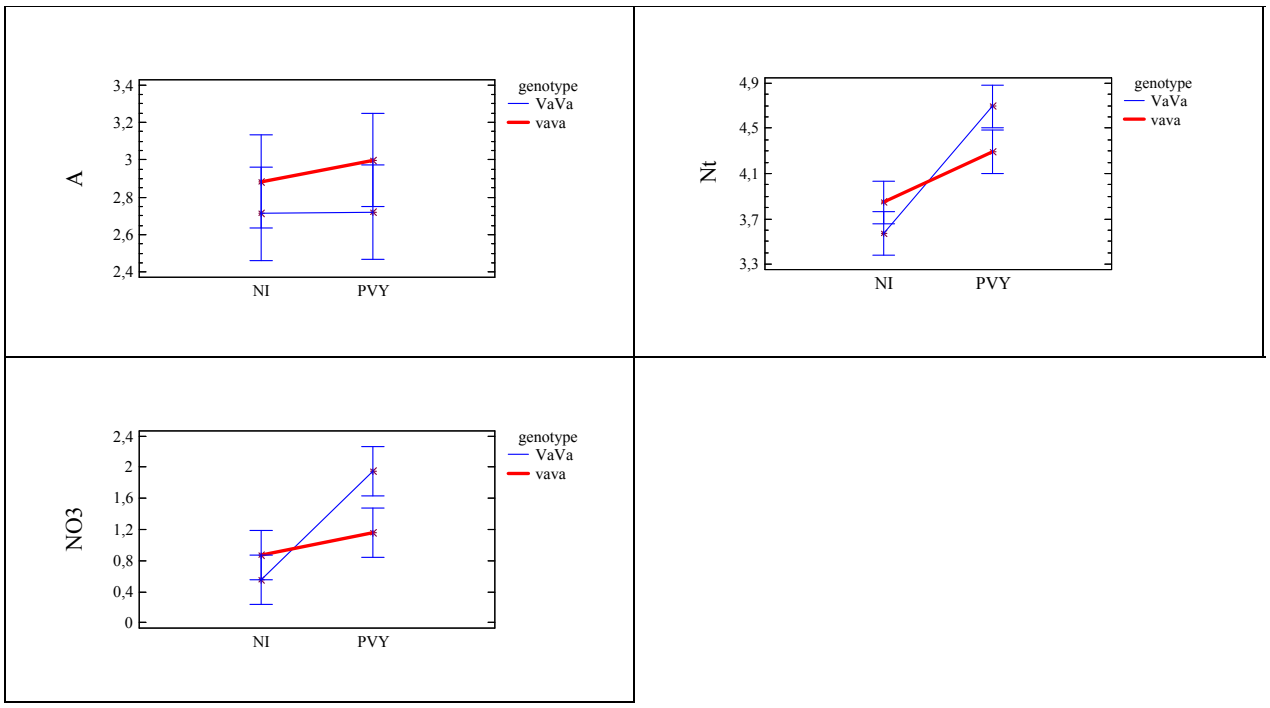
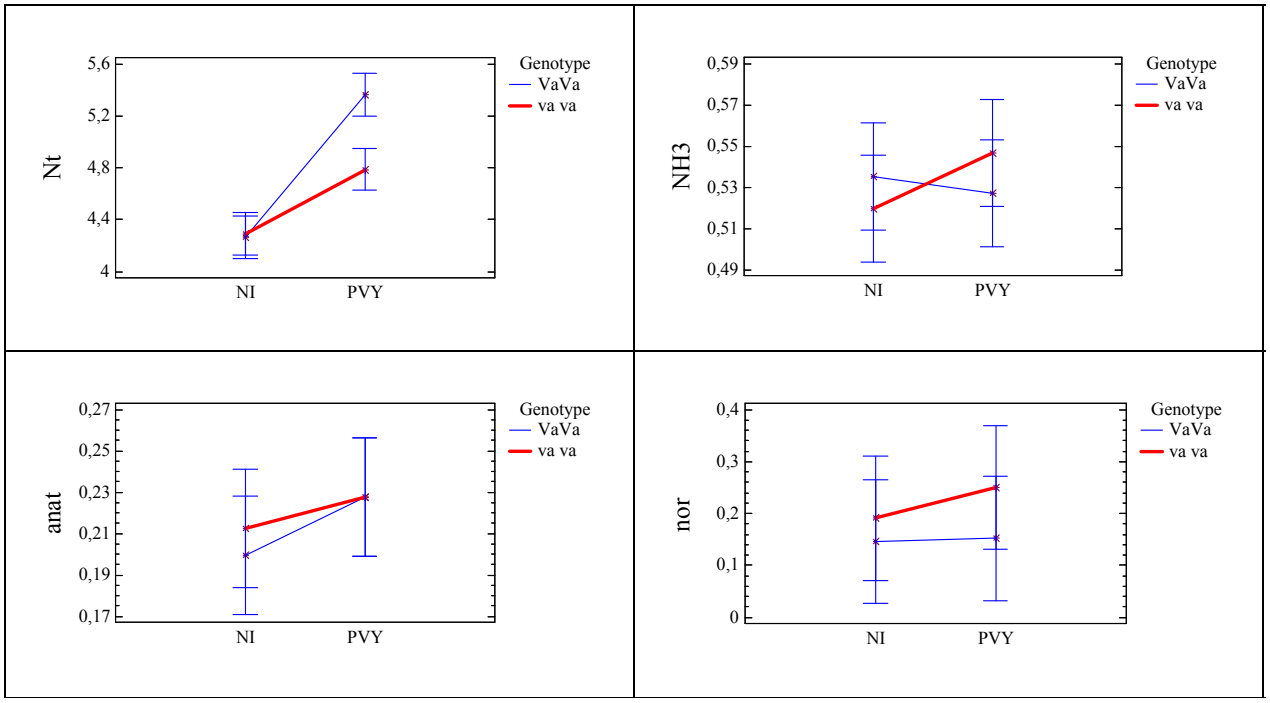


Figure 4. Means and 95% confidence intervals for virus treatment x genotype combinations , data from whole leaves.
(NI: non inoculated, PVY: inoculated with PVY^N. A: total alkaloids, % dm, NIRS estimate. Nt: total nitrogen, % dm, NIRS estimate.
NH3: ammonia, % dm, NIRS estimate. NO3: nitrate, % dm, continuous flow analysis)



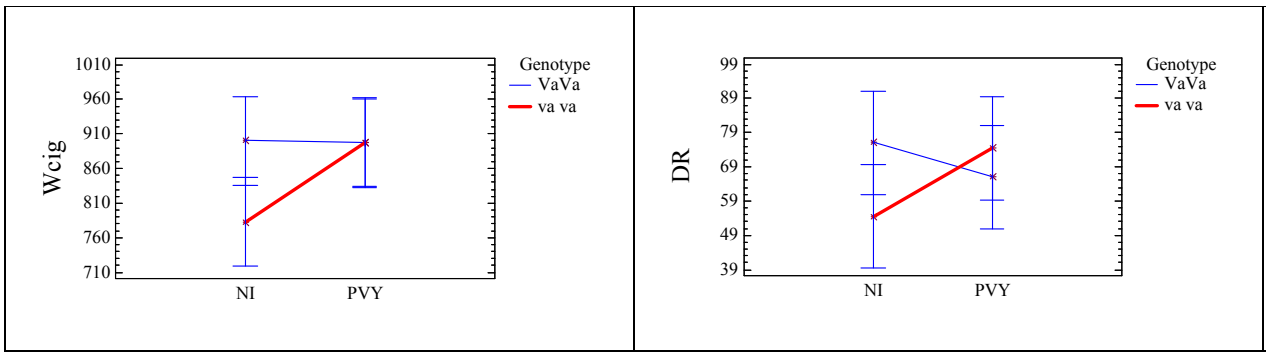


Figure 5. Means and 95% confidence intervals for virus treatment x genotype combinations , data from strips and cigarettes.
Nt: total nitrogen, NH3: ammonia, nic: nicotine, anat: anatabine, nor: nornicotine, all chemical data in % of dry matter
Wcig: mean cigarette weight, mg, DR: mean cigarette draw resistance, mm, Tar: Nicotine Free Dry Particulate Matter yield, mg/cigarette,
niccig: nicotine yield, mg/cigarette

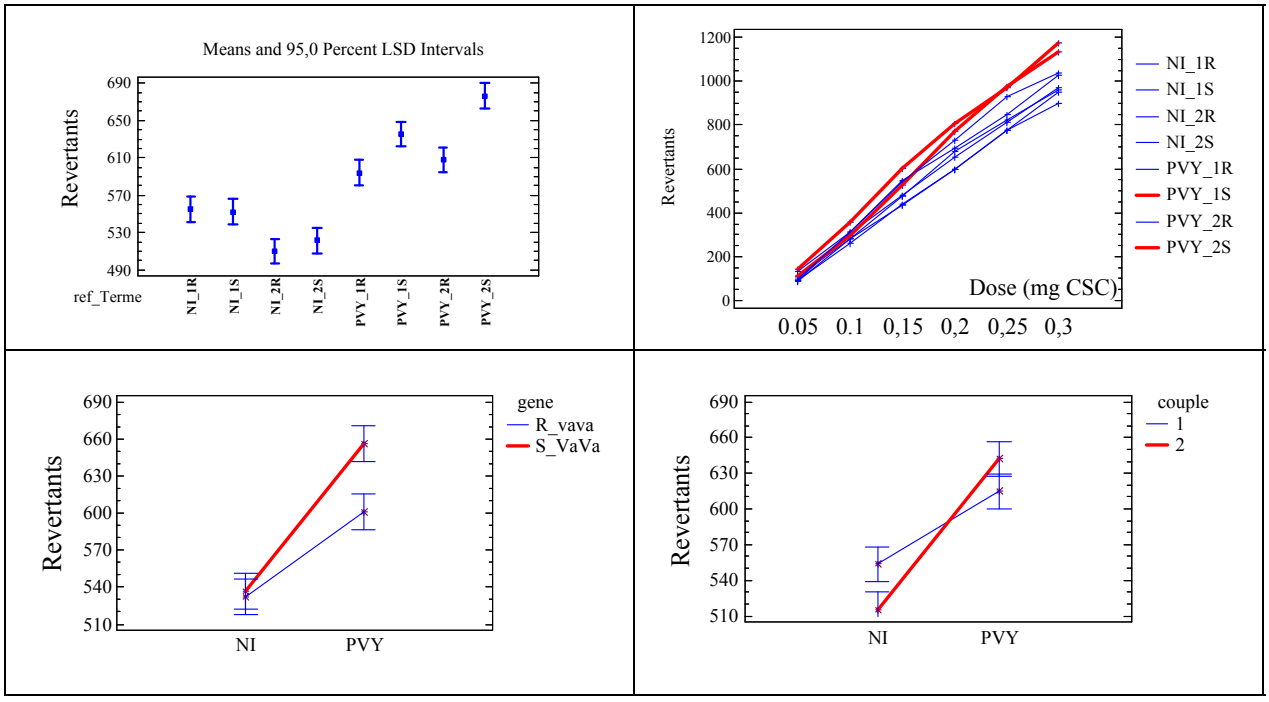


Figure 6. Compared results of the 8 combinations of treatments (PVY inoculated or not, couple of lines and "vava" or VaVa" genotype) for the revertant count means, and interactions.
NI = non inoculated, PVY = PVY^N inoculated,
1R = couple 1, (*va va*) (PVY resistant), 1S = couple 1, (*Va Va*) (PVY susceptible)
2R = couple 2, (*va va*) (PVY resistant), 2S = couple 2, (*Va Va*) (PVY susceptible).

Table 5. Multifactorial variance analysis of the revertant counts according to two models.

1) Revertant count = constant term + CSC dose effect + term effect + Ames test run effect + smoking run effect + two order interactions + residual.
The word "term" in this model refers to the 8 combinations of genotypes, couples and virus treatments.

Analysis of Variance for Revertants - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-
MAIN EFFECTS					
A:Dose	1,89635E7	5	3,7927E6	1716,41	0
B:Term	555381,0	7	79340,1	35,91	0
C:Ames test run	710594,0	1	710594,0	321,58	0
D:Smoking run	14708,8	1	14708,8	6,66	0
INTERACTIONS					
AB	184729,0	35	5277,96	2,39	0
AC	146446,0	5	29289,3	13,26	0
BC	40798,4	7	5828,34	2,64	0
BD	78652,4	7	11236,1	5,08	0
RESIDUAL	271789,0	123	2209,67		
TOTAL (CORRECTED)	2,09666E7	191			

All F-ratios are based on the residual mean square error.

2) Revertant count = constant term + CSC dose effect + virus treatment effect + genotype effect + couple effect + Ames test run effect + smoking run effect + two order interactions + residual.

Analysis of Variance for Revertants - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P
MAIN EFFECTS					
A:Dose	1,89635E7	5	3,7927E6	1467,39	(
B:virus treatment	423893,0	1	423893,0	164,00	(
C:genotype	42497,9	1	42497,9	16,44	(
D:couple	1304,69	1	1304,69	0,50	(
E:Ames test run	710594,0	1	710594,0	274,93	(
F:Smoking run	14708,8	1	14708,8	5,69	(
INTERACTIONS					
AB	102806,0	5	20561,3	7,96	(
AC	28277,8	5	5655,56	2,19	(

AE	146446,0	5	29289,3	11,33	(
BC	30716,7	1	30716,7	11,88	(
BD	51466,6	1	51466,6	19,91	(
CD	4986,78	1	4986,78	1,93	(
DF	26684,5	1	26684,5	10,32	(
RESIDUAL	418715,0	162	2584,66		
TOTAL (CORRECTED)	2,09666E7	191			

All F-ratios are based on the residual mean square error.