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## Occurrence of Killer Yeast Strains in Fruit and Berry Wine Yeast Populations

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### Summary

Apple, cranberry, chokeberry and Lithuanian red grape wine yeast populations were used for the determination of killer yeast occurrence. According to the tests of the killer characteristics and immunity the isolated strains were divided into seven groups. In this work the activity of killer toxins purified from some typical strains was evaluated. The analysed strains produced different amounts of active killer toxin and some of them possessed new industrially significant killer properties. Total dsRNA extractions in 11 killer strains of yeast isolated from spontaneous fermentations revealed that the molecular basis of the killer phenomenon was not only dsRNAs, but also unidentified genetic determinants.

*Key words:* dsRNA, immunity, killer activity, *Saccharomyces cerevisiae*, wine yeast

### Introduction

The production of yeast killer toxin is a well-established phenomenon among many yeast genera and species (1,2). The killer activity is readily detectable only when a suitable sensitive strain is tested. There is evidence that killer yeasts may secrete different killer toxins with activities specific for different yeast target cells (3,4).

In *Saccharomyces* three different killer toxins (K1, K2 and K28) have clearly been identified, which are all genetically encoded by double-stranded M-dsRNA »killer« viruses persistently existing within the cytoplasm of the infected host cell (4). Members of all three groups are capable of killing non-killer yeasts as well as killer yeasts of the opposite killer class, but they remain immune to their own toxin and to the strains belonging to the same killer group. All three killer toxin-coding M-dsRNAs differ in size (M1 1.8 kb, M2 1.5 kb, M28 2.0 kb) and show little, if any, sequence homology (5–7). In addition, M-dsRNA-containing viruses are defective particles that

depend on a larger dsRNA species, denoted L-A (4.6 kb), which is also cytoplasmically inherited and encapsidated in virus-like particles (VLP). L-A encodes the capsid found in M and L-A VLPs and it also encodes the capsid-polymerase fusion protein found as a minor component of these VLPs (4). Cells containing neither of the dsRNAs or only L-A dsRNA are sensitive non-killers.

Recently two new killer types which are encoded on the chromosomal DNA of *S. cerevisiae* have been described. These killer activities were weaker than those of the known killer strains (8).

K1 and K2 have virtually identical toxin activities: both bind to a  $\beta$ -1,6-D-glucan component in the walls of sensitive cells and then disrupt the ion exclusion barrier of the target cell plasma membrane (9–11). The third killer, K28, binds to the  $\alpha$ -1,3-linked mannose residues in the mannoprotein of the walls of sensitive strains, and

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the earliest observable effect of K28 toxin on sensitive cells is a reversible inhibition of DNA synthesis (7,12).

Soon after the discovery of the viral killer system in *Saccharomyces*, killer strains were also found in the genera *Debaryomyces*, *Pichia*, *Kluyveromyces*, *Candida*, *Cryptococcus*, *Ustilago* and *Williopsis* (1,12–14).

Whereas the genetic basis of the *Kluyveromyces lactis* killer phenotype was shown to be associated with the presence of two dsDNA plasmids which could be transformed and steadfastly expressed in several different yeast genera (15), the molecular basis in all other killer yeasts is either dsRNA, or unknown, or thought to be chromosomally encoded (12,14).

The occurrence of killer character among grape wine yeasts in a number of winemaking countries was studied (16–18). In the previous work we reported the results of a study of killer strains among Lithuanian yeast. According to their cross-reactions and pH optimum we divided them into groups (19). In this paper the most recent data on investigations of killer activity and molecular basis of the killer phenomenon in strains isolated from spontaneous fruit and berry wine fermentation were summarised.

## Materials and Methods

### Strains and media

The yeast strains used in this work were obtained from winery »Anyksciu vynas« and cooperative society »Vaisiu sultys«. The raw materials used were obtained from the Baltic countries and Russia, which is why yeast infection was not investigated. Different *S. cerevisiae* strains have been used in winemaking factories for 10 years. From the fermenter lees which were formed after 16 fermentations containing 1560 hL, 100 probes of yeast were taken. Pure yeast culture was isolated by repeated cloning.

The *S. cerevisiae* strain  $\alpha$ 1 (MAT $\alpha$  leu2-2 (KIL-0)), sensitive to all killers (20), was used for testing killer toxin activity. For immunity tests we used *S. cerevisiae* killer strains: K7 (MAT $\alpha$  arg9 (KIL-K1)) (21), Rom-K100 (wild type, HM/HM (KIL-K2)) (22), M437 (wild type, HM/HM (KIL-K2)) (23) and MS300 (MAT $\alpha$  leu2 ura 3-52 (KIL-K28)) (24).

The identification of yeast strains was done in the Microbiological Laboratory of Lithuanian Public Health Centre. Automatised mini AP I 20 CAUX system for clinical yeast identification was used applying methods and reagents of the worldwide recognised Merieux Foundation (France).

Yeast cells were grown in YEPD medium containing 1 % yeast extract, 2 % peptone and 2 % glucose. Buffered methylene blue medium (MB) containing YEPD adjusted to the required pH (using 0.2 mol/L citrate-phosphate buffer) with 0.03 % methylene blue incorporated (23) was used for killer activity and immunity tests.

### Assay of killer and immunity phenotypes

#### Plate test

The tested yeast strains were spotted on the MB agar plates seeded with a sensitive strain  $\alpha$ 1. After the incu-

bation of plates at 26 °C for 3–5 days, clear zones of growth inhibition surrounding the killer cells were evaluated. The size of the growth inhibition zone was interpreted as a killer activity.

The sensitivity/immunity tests were performed by patching colonies of killer strains onto the MB plates with an overlay of the yeast strain of interest (approximately  $10^6$  cells per plate).

#### Well test of killer activity

Killer toxins were prepared by growing yeast strains in a liquid MB medium without methylene blue at pH=4.4 and pH=5.2 for 96 h at 20 °C. Yeast cells were isolated by centrifugation at 3000  $\times$  g for 10 min at 4 °C and the supernatant was filtered through sterile 0.22- $\mu$ m polyvinylidene fluoride membrane. The activity of killer toxins was tested using lysis zone assay by pipetting 0.1 mL of the resulting supernatant in wells (10 mm in diameter) cut into the agar. The diameter of the growth-free zone around the wells is proportional to the logarithm of the killer toxin activity. Killer toxin activity was calculated according to the formula  $D=(5 \cdot \log A) \cdot 10$ , where D is growth-free zone in mm and A is toxin activity in relative units (U/mL)(24).

### dsRNA preparation

dsRNAs were prepared from yeasts that had been grown until the late logarithmic/early stationary phase at 30 °C in YEPD medium according to the procedure described by Fried and Fink (25) with the following modifications. In order to quantitatively remove proteins from the nucleic acids the crude cell extract was incubated and gently shaken for 30 min in 0.05 mol/L Tris-H<sub>2</sub>SO<sub>4</sub> (pH=9.3) containing 2.5 % 2-mercaptoethanol and for 1.5 h in solution containing 0.1 mol/L NaCl, 0.01 mol/L Tris-HCl (pH=7.5), 0.01 mol/L Na<sub>2</sub>EDTA, 0.2 % sodium dodecyl sulphate and an equal volume of bidistilled phenol. After 10 min of centrifugation at 5000  $\times$  g the aqueous phase was mixed with an equal volume of chloroform. Nucleic acids were recovered from the aqueous phase by precipitation with about 2.5 volumes of 96 % ethanol and stored at –20 °C for 24 h. After 15 min of centrifugation at 16 000  $\times$  g the pellet was dissolved in 400  $\mu$ L of TE buffer (0.01 mol/L Tris-HCl (pH=7.5) and 0.001 mol/L Na<sub>2</sub>EDTA). Partially purified dsRNAs were analysed by electrophoresis on 1 % agarose gels and stained with ethidium bromide (0.5  $\mu$ g/mL).

## Results and Discussion

Apple, cranberry, chokeberry and Lithuanian red grape wine yeast populations were used for the determination of killer yeast occurrence. Killer ability was tested in 16 962 yeast strains. Spreading of killer yeast in wine yeast populations depended on the sort and quality of raw material. Killer yeast in apple wine yeast population included from (0.09 $\pm$ 0.04) to (46.61 $\pm$ 3.23) %, among which (0.93 $\pm$ 0.10) % were intense killers. In Lithuanian red grape wine (6.23 $\pm$ 0.46) % of the yeast population were killers and (2.51 $\pm$ 0.31) % were intense killers, while in cranberry wine (67.50 $\pm$ 0.93) % of yeast population were killers and (6.51 $\pm$ 0.47) % were intense killers. Chokeberry wine yeast population contained (0.10 $\pm$ 0.05) % of poor

killers. Selected strains retained their killer abilities throughout 2 years while going through repeated passages and incubated at 25 and 30 °C. According to the tests of killer characteristics and immunity, we divided the tested strains into seven groups. These groups showed an optimum of toxin activity at different acidic pH values: group I at pH from 4.0 to 4.8, group II at about pH=5.2, group III at pH from 4.0 to 4.8, group IV at pH from 4.8 to 5.2, group V at pH from 3.6 to 4.4, group VI at pH from 4.0 to 4.4, and group VII at pH from 4.4 to 4.8. It was determined that the tested yeast strains produced different amounts of killer toxins (Table 1). Strains that produce the largest amount of killer toxin were put into the first group. All the found yeast killer clones were compared with *S. cerevisiae* sensitive strains and

with strains of K1, K2 and K28 killer systems, which are employed only in fruit-berry winemaking. Other groups were formed according to the decrease of killer toxin secretion. It is interesting to indicate that we isolated typical K2 killers as well as new  $K_N$ ,  $K_Z$  and  $K_X$  strains.  $K_N$  are potentially new possible killers, compared with *S. cerevisiae* killer systems of K1, K2 and K28 types.  $K_N$  killers showed immunity to K2 type toxins, at the same time killing not only K1 and K28, but also K2 type killers too. Type  $K_Z$  was active against K28 and resistant to all type killer toxins.  $K_X$  strains did not kill K1, K2 and K28 killers but killed sensitive strain  $\alpha$ '1 only.

For estimation of a level of produced active toxin, killer strains were grown for 96 h in liquid MB media without methylene blue. It was determined that members of the group I, Apple 7-2.1, Apple 1-1 and Apple 3-2, produced high levels of secreted killer toxin, *i.e.* 199.5, 158.5 and 100 U/mL, respectively. The wild type K2 killer expressing strain M437 produced 125.9 U/mL of toxin, whereas another wild type strain Rom-K100 produced lower level of killer toxin, *i.e.* 79.4 U/mL.

Colonies of the strain Apple 2-1 (group II) showed moderate killer activity on the lawn of sensitive strain  $\alpha$ '1 (plate test, Table 1). Surprisingly, for unknown reasons, the level of toxin accumulated in supernatants from the same strain was insufficient to kill  $\alpha$ '1 in well assay (Fig. 1).

Strains isolated from cranberry and Lithuanian red grape fell into the group III. All of them possessed mild killer activity in plate tests as well as in well assay (Tables 1 and 2). It was determined that Cranberry 1 and Grape 1 produced about four times lower amount of active toxin (15.8 and 25.1 U/mL, respectively) in comparison with wild type killer Rom-K100. Group III needs

Table 1. Killer properties of some tested yeast strains

Groups	Strains	Optimal pH	Killer phenotype (plate test) <sup>1</sup>	Killer type
Standards	K7	4.6–4.8	+++	K1
	Rom-K100	4.0–4.4	+++±	K2
	M437	4.0–4.4	++++	K2
	MS300	5.0–5.8	+++	K28
I	Apple 7-2.1	4.0–4.8	+++++	K2
	Apple 1-1	4.0–4.8	++++	K2
	Apple 3-2	4.0–4.8	+++	K2
II	Apple 2-1	5.2	±	$K_N$
III	Cranberry 1	4.0–4.8	++	$K_Z$
	Cranberry 2	4.0–4.8	+	$K_Z$
	Grape 1	4.0–4.8	++	$K_N$
	Grape 2	4.0–4.8	+	$K_N$
IV	Apple 5-3.1	4.8–5.2	+	$K_X$
	Apple 7-4.2	4.8–5.2	+	$K_N$
	Apple 9-1	4.8–5.2	+	$K_N$
	Apple 8-1.2	4.8–5.2	±	$K_X$
V	Apple 1-5	3.6–4.4	+	$K_X$
	Apple 2-2	3.6–4.4	+	K2
	Apple 2-5	3.6–4.4	±	K2
VI	Apple 8-1N	4.0–4.4	±	K2
	Grape 1N	4.0–4.4	±	$K_Z$
	Grape 2N	4.0–4.4	±	$K_Z$
	Apple 9-3	4.0–4.4	+	$K_Z$
	Apple 10-1	4.0–4.4	+	$K_X$
	Chokeberry 1N	4.0–4.4	±	$K_X$
VII	Apple 4-2.1	4.4–4.8	s	$K_X$
	Apple 4-3.1	4.4–4.8	s	$K_X$
	Apple 7-1.1	4.4–4.8	s	$K_X$

<sup>1</sup> Zone of inhibition (width in mm): +++++ (≥9), ++++ (9–7), +++± (7–6), +++ (6–5), ++± (5–4), ++ (4–3), ± (3–2.5), + (2.5–1.5), ± (1.5–1), s (1–0); K2 – K2 killer phenotype;  $K_N$  – new killer phenotype (tested strain killed K1, K2 and K28 type killer strains);  $K_Z$  – new killer phenotype (tested strain killed only K28 type killer strains);  $K_X$  – new killer phenotype (tested strain killed only sensitive strain  $\alpha$ '1)

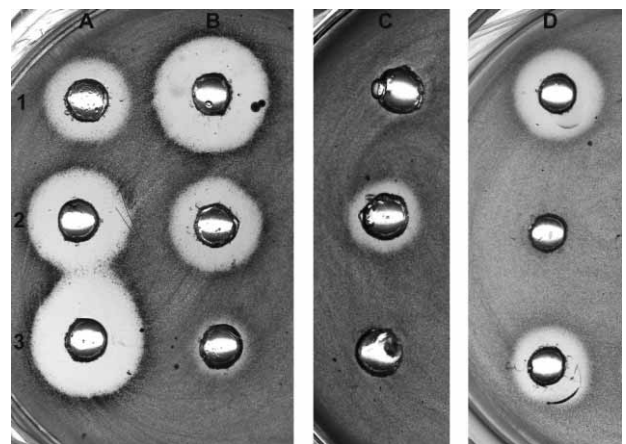


Fig. 1. Well test of killer activity. Killer toxin activities were determined in supernatants of cultures by pipeting 100  $\mu$ L of samples into wells (10 mm in diameter) cut into MB agar plates seeded with the sensitive  $\alpha$ '1 yeast strain and incubating the plates at 20 °C for 96 h. Diameter of the growth-free zone around the wells is proportional to the logarithm of the killer toxin activity, which is expressed in arbitrary units (U/mL). Column A: 1, strain Rom-K100; 2, strain M437; 3, strain Apple 7-2.1. Column B: 1, strain Apple 1-1; 2, strain Apple 3-2; 3, strain Apple 1-5. Column C: 1, strain Cranberry 1; 2, strain Grape 1; 3, strain Grape 2N. Column D: 1, strain MS300; 2, strain Apple 2-1; 3, strain Apple 5-3.1

Table 2. Killer toxin activities

Groups	Strains	Zone of inhibition / mm (well test)	Activity of killer toxin/ (U/mL)
Standards	Rom K-100 [K2]	4.5	79.4
	M437 [K2]	5.5	125.9
	MS300 [K28]	5.0	100.0
I	1. Apple 7-2.1 [K2]	6.5	199.5
	2. Apple 1-1 [K2]	6.0	158.5
	3. Apple 3-2 [K2]	5.0	100.0
II	Apple 2-1 [K <sub>N</sub> ]	–	–
III	1. Cranberry 1 [K <sub>Z</sub> ]	1.0	15.8
	2. Grape 1 [K <sub>N</sub> ]	2.0	25.1
IV	1. Apple 5-3.1 [K <sub>X</sub> ]	3.5	50.1
	2. Apple 7-4.2 [K <sub>N</sub> ]	0.5	12.6
	3. Apple 9-1 [K <sub>N</sub> ]	–	–
V	Apple 1-5 [K <sub>X</sub> ]	1.5	19.9
VI	Grape 2N [K <sub>Z</sub> ]	0.5	12.6

According to the results of identification:

group I: 1. *Saccharomyces cerevisiae* 99.7 %  
2. *Saccharomyces cerevisiae* 99.7 %  
3. *Saccharomyces cerevisiae* 99.7 %

group II: *Saccharomyces cerevisiae* 99.7 %

group III: 1. *Candida valida* 73.9 %  
*C. incons./norvegen.* 24.6 %  
2. *Candida holmii* 75 %  
*C. incons./norvegen.* 24.6 %\*

group IV: 1. *Candida holmii* 95 %  
2. *Saccharomyces cerevisiae* 99.7 %  
3. *Candida holmii* 90.6 %

group V: *Saccharomyces cerevisiae* 99.7 %

group VI: *Saccharomyces cerevisiae* 99.7 %

\* The species of that yeast cannot be identified by this method. The culture was pure

special grade identification because it aspires to new quality/qualities (Table 2).

The observed pH optimum for killers from the group IV was unusually high, 5.2 (Table 1). Therefore, for an estimation of a level of secreted toxin these strains were grown in liquid media at pH=5.2. Data of killer activity assay were compared with wild type K28 killer expressing strain MS300, most active at the same pH. The killer activity of strain Apple 5-3.1 reached 50.1 U/mL, *i.e.* 50 % of the activity of MS300. Apple 7-4.2 produced barely detectable amount of toxin (12.6 U/mL). At the same time secretion of poor killer Apple 9-1 (Table 1) was indeterminable in the well assay (Table 2). After the identification, it was determined that all K<sub>N</sub> killers were strains of *S. cerevisiae* and needed cloning and sequencing of killer gene.

The tested strains from the remaining groups V–VII showed only weak secretion of killer toxin (Table 1). We chose the most typical examples, Apple 1–5 and Grape 2N, for well analysis. The comparison of the amounts of produced toxin in the wild type strain Rom-K100 and the mentioned strains indicated that the tested strains were able to produce about 25 and 15 %, respectively, of wild type strain toxin (Table 2, Fig. 1).

The killer yeasts, belonging to *Saccharomyces cerevisiae* species, have been classified into three main groups (K1, K2 and K28) on the basis of the molecular characteristics of the secreted toxins, their killing profiles, the lack of cross-immunity and the encoding genetic determinants (1). In our work all isolated strains were classified into appropriate killer types according to the tests of the killer characteristics and immunity. Not only typical K2 killers, but also unconventional killer types K<sub>N</sub>, K<sub>X</sub> and K<sub>Z</sub> were found. In order to specify the dependence of the tested strains to particular killer type, the analysis of dsRNA was performed.

The patterns of cytoplasmic dsRNAs isolated from some of the strains used in this work (Table 1) are illustrated in Fig. 2, an agarose gel with approximately equal amounts of dsRNA loaded in each lane. All of the L-dsRNAs had similar mobilities and therefore could not be distinguished from the L species of the representative *S. cerevisiae* K1, K2 and K28 killer strains. It is known that K1, K2 and K28 killer phenotypes are associated with the presence of the corresponding M-dsRNA species (4).

We demonstrated that standard killer strains K7 (type K1), M437 (type K2) and MS300 (type K28) contained a toxin-coding M-dsRNAs 2.0 kb, 1.8 kb and 2.2 kb, respectively. M-dsRNAs isolated from group I killer strains Apple 7-2.1, Apple 1-1 and Apple 3-2 showed similar

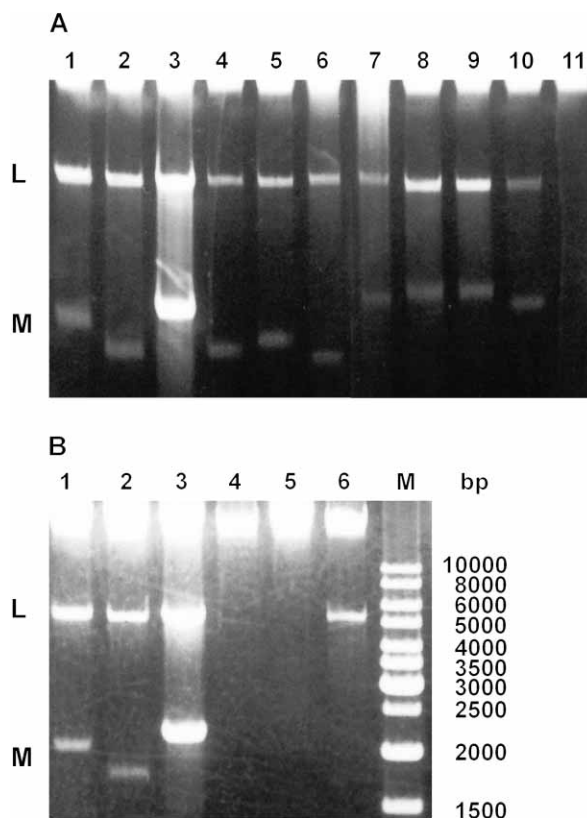


Fig. 2. Agarose gel electrophoresis of dsRNA extracts from yeasts. A: lane 1, strain K7; lane 2, strain M437; lane 3, strain MS300; lane 4, strain Apple 7-2.1; lane 5, strain Apple 1-1; lane 6, strain Apple 3-2; lane 7, strain Apple 2-1; lane 8, Apple 5-3.1; lane 9, Apple 7-4.2; lane 10, Apple 9-1; lane 11, Apple 1-5. B: lane 1, strain K7; lane 2, strain M437; lane 3, strain MS300; lane 4, strain Cranberry 1; lane 5, strain Grape 1; lane 6, strain Grape 2N; M, size marker (Gene Ruler™ DNA Ladder Mix, MBI Fermentas)

mobility like M2 dsRNA (1.8, 1.85 and 1.75 kb, respectively, Fig. 2). According to the killer activity and immunity tests these strains had been described as K2 type killers previously and dsRNA electrophoretical analysis supported these results.

Strains from groups II and IV were characterised as  $K_N$  and  $K_X$  type killers (Table 1). They showed maximum toxin activity at pH=5.2, specific to K28 killer toxin. dsRNAs from strains Apple 2-1, Apple 5-3.1, Apple 7-4.2 and Apple 9-1 were isolated and analysed electrophoretically. The size of all M-dsRNAs was identical to M28 dsRNA from the strain MS300 and reached 2.25 kb (Fig. 2). These findings enabled us to suppose that the mentioned killer strains could be derivatives of K28 killer type with altered killer and immune properties.

It is interesting to point out that in mild killer strains isolated from cranberry and Lithuanian red grape (Cranberry 1 and Grape 1) we were not able to find neither M- nor L-dsRNAs, whereas in the strain Grape 2N only L-dsRNA was detected (Fig. 2). We suggest that in these cases killer phenotype could be encoded on chromosomal DNA. It is possible that strain Apple 1–5 possesses the same genetic determinants, because dsRNAs were also not found (Fig. 2).

## Conclusions

Among the killer yeasts isolated from spontaneous fruit and berry wine fermentation a wide range of killer properties was described. The analysed strains produced different amounts of active killer toxin and some of them possessed new industrially significant killer properties. They could be recommended for fermentation of particular types of wine – apple, cranberry, chokeberry or grape. We suggest that strong killers isolated from apple wine lees (Table 1, group I) may be effective in suppressing wild yeast strains during fermentation, and there is also the possibility of engineering broad-range killer yeast strains to control strains from other genera. We may also infer, from the results of this study, that the tested killers differ in the genesis of genetic determinants. Common killer phenotype was determined by dsRNA molecules. However, in some cases dsRNAs were not detected, although the tested strains produced active toxins. Further experiments will reveal the molecular basis of this killer phenomenon.

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## Postojanje sojeva ubilačkih kvasaca u kvascima vina od bobičastog voća

### Sažetak

Da bi se odredila prisutnost ubilačkih kvasaca, upotrijebljena je populacija kvasaca na jabukama, brusnicama, crnoplodnim aronijama i litavskom crvenom grožđu. Prema ubilačkim značajkama i imunološkim svojstvima sojevi su podijeljeni u sedam skupina. Utvrđena je aktivnost ubilačkih toksina izoliranih i pročišćenih iz nekih tipičnih sojeva. Analizirani sojevi proizvode različitu količinu aktivnih ubilačkih toksina, a neki od njih imaju nova industrijski značajna ubilačka svojstva. Ukupnom ekstrakcijom dsRNA iz 11 ubilačkih kvaščevih sojeva, izoliranih tijekom spontanijeh fermentacija, otkriveno je da molekularna baza ubilačkog fenomena nisu samo dsRNA nego i neidentificirane genetičke determinante.