PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/54, 38/44

A1

(11) International Publication Number: WO 95/08346

(43) International Publication Date: 30 March 1995 (30.03.95)

(21) International Application Number: PCT/US94/10082

(22) International Filing Date: 8 September 1994 (08.09.94)

(30) Priority Data:
107,080 23 September 1993 (23.09.93) IL

(71) Applicant (*for all designated States except US*): YISSUM RESEARCH DEVELOPMENT CO. OF THE HEBREW UNIVERSITY OF JERUSALEM [IL/IL]; P.O. Box 4279, Jerusalem (IL).

(71) Applicant (*for VN only*): COHEN, A., David [US/IL]; P.O. Box 60, 84100 Beer-Sheva (IL).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): SHOSEYOV, Oded [IL/IL]; 5 Erez Street, 99797 Karmei Yosef (IL). REIZ, Levava [IL/IL]; 12 Rahavat Ilan Street, 54056 Givat Shmuel (IL). OZERI, Uzi [IL/IL]; D.N. Tzefon Yehuda, 90938 Kibbutz Rosh Zurim (IL). BRAVDO, Ben-Ami [IL/IL]; 11 Hankin Street, 76354 Rehovot (IL). GOREN, Raphael [IL/IL]; 37 Menucha Ve'Nachala Street, 76247 Rehovot (IL).

(74) Agent: NEIMARK, Sheridan; Browdy and Neimark, Suite 300, 419 Seventh Street, N.W., Washington, DC 20004 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A METHOD FOR THE BIOLOGICAL CONTROL OF POLLEN IN PLANTS

(57) Abstract

A method for the biological control of pollen in plants, including the thinning of fruits, reducing seeds in citrus plants and reducing the incidence of shotberries in grapes, by applying an effective amount of activated or inactivated RNase or mixtures of these to the flowers of the plants.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

A METHOD FOR THE BIOLOGICAL CONTROL OF POLLEN IN PLANTS

BACKGROUND OF THE INVENTION

The present invention relates to a method of the biological control of pollen in plants using RNase. The present invention particularly relates to the thinning of a large variety of fruit plants, the reduction of seeds in citrus plants and the reduction of the incidence of shotberries in grapes using activated RNase, inactivated RNase or mixtures of these.

The purpose of thinning fruit, whether it is done manually, chemically or mechanically, is to reduce crop load early in the fruit development period to maximize return of bloom (for apple) and ensure attainment of commercially acceptable fruit size (for peach, nectarine, plum, apple and table grapes). Although apples and grapes are routinely thinned in many districts, satisfactory results have not always been consistent, because sensitivity to any specific thinning agent varies from clone to clone, and climatic conditions enhance or diminish the efficiency of the chemicals. In peach and nectarines hand thinning is the only practical method that is currently employed, simply because none of the many chemicals and cultural practices employed, give consistent results. Among the many methods are: Control of flower buds initiation by gibberellic acid (M.Sc. Thesis, A. Breuer-Mizrahi, (1991) The Hebrew University of Jerusalem), flower thinners (R. Gaash, S. Lavee, A. Golan, D. Brown, "Alon Hanotea", (1969), pp. 319-325), fruitlet thinners (S. Morini, G. Vitagliano, C. Xiloyannis, J. Am. Soc. Hort. Sci., 40, (1976), pp. 237-247, and G. E. Stembridge, C. G. Gambrell, Ibid., 96, (1971) pp. 7-9), urea, as well as cold water.

Two major problems are associated with these methods:

1. Thinning intensity was inconsistent and difficult to control.
2. The broad range of effect makes it impossible to determine a precise developmental stage (of the flower or the fruitlet) for optimal treatment

In grapes, gibberellic acid is routinely used for thinning Tompson cultivar. The results, however, require always hand thinning for adjustment. The same treatment in Perlette is not practiced for the last 15 years, since it was found that in this variety, the gibberellic acid causes irregular berry size (shortberries), therefore hand thinning is practiced in Perlette.

The series of events that occur between pollination and fertilization include processes of specific recognition of pollen grains and pollen tubes by the pistil tissues. The control of the pistil on pollen germination and pollen tube growth has been mainly studied in self-incompatible systems, in which arrest of pollen tube is the consequence of a contact between pollen and pistil of the same genotype. In self-compatible matings there is some evidence that the pollen tube is directed toward the ovule by trophic and chemotropic effect in the style. However, there is still a paucity of information about the female control of the male gametophyte. Most economically important apple ((Malus domestica) cultivars require cross-pollination for fruit set. Peach (Prunus persica) is known as self-compatible. In these species the growth of pollen tubes in the style has been thoroughly investigated by physiological and histochemical studies. It has been established that the kinetics of pollen tube growth is controlled by each part of the gynoecium, starting with the stigma and ending with the ovule. However, the nature of this control, or any stylar product in which it is accomplished by, have not been characterized in these works.

Recently a major stylar protein (S-protein) was isolated from the self-incompatible species *Nicotiana alata*. The gene that encodes for the protein was cloned and sequenced and was found to share homology with fungal RNase. It was established that this protein is indeed RNase, and was found to arrest pollen tube growth (B. A. McClure et al., Nature, 1989, pp. 955-957). However, the RNase was also found in stylar diffusates of self-compatible species such as *Nicotiana tabacum* (McClure et al., 1989) and more recently in peach (Roiz and Shoseyov, 1993, unpublished data) and citrus (Roiz, Shoseyov and Goren, 1993, unpublished data). It is speculated that the RNase penetrate the pollen tube, degrade ribosomal and mRNAs and thereby cause its abortion.

Closely related to the thinning of the fruits is the reduction of seeds in citrus plants, which is very desirable from a practical as well as commercial point of view. Until now there is no known reliable method for reducing the seeds in citrus plants.

Similarly, there is no known reliable method for reducing the incidence of shotberry in grapes.

RNase is a known enzyme obtainable from a large variety of sources. One main source is the fermentation of various fungi.

SUMMARY OF THE INVENTION

In accordance with the present invention there is provided a method for the biological control of pollen in plants, comprising applying an effective amount of activated or inactivated RNase or a mixture thereof. The present invention results in the thinning of a large variety of fruit plants, the reduction of seeds in citrus plants and, in the case of grapes, the reduction of the incidence of shotberries.

DETAILED DESCRIPTION OF THE INVENTION

RNase may be obtained from sources such as fungi, bacteria, plants, animal and viral. When obtained from fungal sources, the RNase may easily be obtained by the fermentation of various fungal sources. Examples are Aspergillus niger, Aspergillus claritus, Aspergillus oryzae and Rhisopus miveus, preferably Aspergillus niger and Aspergillus oryzae. A most preferred fungus is Aspergillus niger B1(CMICC Number 324,626). This strain was deposited with the Commonwealth Mycological Institute, Ferry Lane, Kew, Richmond-upon-Thames, Surrey TW9 3A5, United Kingdom, on 20 May, 1988.

The plants where the method of the biological control of pollen is chosen from the group consisting of fruit trees, ornamental trees, vegetables, field crops, plantations, ornamentals and the like. The trees where the method of thinning was found to be applicable are chosen from the group consisting of deciduous plants, tropical plants, sub-tropical plants, citrus plants, nut tree and grape vines. Examples of deciduous plants are stone fruit, pears, apples, quince, peaches, plums, nectarines, cherries and apricots.

Examples of ornamental trees are olive and Fica trees.

Examples of field crops are cereals, especially hybrids.

Examples of citrus plants where the RNase in use in the thinning and the reduction of seeds are orange, lemon, grapefruit, clementina, mandarin, citron, pomela and their hybrids.

The method of the prevention of fruit set of field crops of the present invention was found to be especially useful in preventing fruit set of ornaments whose fruits and flowers cause environmental problems.

The RNase is added to the pollen in the form of crude or purified extract of the RNase in the form of spray, powder and the like. A really novel way to apply the RNase is to powder bees and let them distribute the RNase powder during their feeding on the pollen in the flowers.

The RNase is applied at a rate of 1 to 10,000 units per hectare.

While the invention will now be described in connection with certain preferred embodiments in the following examples, it will be understood that it is not intended to limit the invention to these particular embodiments. On the contrary, it is intended to cover all alternatives, modifications and equivalents, as may be included within the scope of the invention as defined by the appended claims. Thus, the following examples, which include preferred embodiments, will serve to illustrate the practice of this invention, it being understood that the particulars shown are by way of example and for purposes of illustrative discussion of preferred embodiments of the present invention only and are presented in the cause of providing what is believed to be the most useful and readily understood description of procedures, as well as of the principles and conceptual aspects of the invention.

EXAMPLE 1

Growth Conditions Of *Aspergillus niger*

Aspergillus niger was grown in medium that contained 1% wheat flour and 0.05% $(NH_4)_2SO_4$ in distilled water. The solution was brought to pH 3.5 with HCl and then autoclaved. An inoculum of 10^6 spores of *A. niger* were suspended into 100 ml of the growth medium and the suspension was incubated at 30°C and shaken at 150-200 rpm. RNase activity in the growth medium reached its peak after 5-7 days of incubation. The medium was then passed through a millipore filter of 0.2 μm to remove any traces of hypha and spores. The medium was ultrafiltered to concentrate the enzyme.

EXAMPLE 2

RNase Assays

RNase activity in the growth medium of *A. niger* was determined by diffusion plate assay, using 0.1% torula yeast RNA (Sigma) in 1% agarose as a substrate. A drop of 10 μl was applied on the substrate in a 15cm petri dish and then was incubated at 37°C for one hour. The substrate was then stained with 0.02% toluidine blue in water. RNase activity was indicated as a white halo on a blue background.

A quantitative spectrophotometric assay of RNase was conducted as a modification of the procedure described by Brown & Ho (1986). Each sample contained 100μl of the examined enzyme mixed with and 4 μg torula yeast RNA and 1 ml of 20 mM buffer of choice. The sample was incubated in 37° for 30 minutes.

The reaction was stopped by putting the tube into ice and adding 200 μl "stop reagent" (0.75% uranyl sulphate in 25% $HClO_4$). The solution was centrifuged in 12,000 rpm for 5 minutes. The supernatant was diluted to 1/20 in distilled water and OD in 260 nm was examined.

The blank solution contained the same component and incubated by the same procedure, only the enzyme was added after the reaction was stopped. The results were given in units/ml, equivalent to standards of bovine pancreatic RNase.

EXAMPLE 3

Optimization of RNase Activity

The optimal pH for RNase activity in the growth medium was examined using 20 mM citrate buffer at the pH value of: 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0. The above spectrophotometric procedure showed that the optimal pH for the fungal RNase activity was 3.5 (Fig. 1).

The effect of presence or lack of metal ions on RNase activity was also examined. Each of the following salts: $MgCl_2$, $FeCl_3$ or $CaCl_2$ was supplemented into the reaction mixture to a final concentration of 10 mM. Additional samples contained the same concentration of DTT as a reductant and EDTA that has strong affinity to metal ions. RNase activity was examined by the above diffusion plate assay. No difference in the diameter of the white halo (1cm) on the blue background was observed between the different treatments and the control.

EXAMPLE 4

The Effect of RNase on Pollen Germination and Pollen Tube Growth in Vitro

Pollen grains of peach cv. Texas were germinated in vitro in growth medium containing 15% (w/v) sucrose, 100μg/ml H_3BO_3, 200μg/ml $MgSO_4.7H_2O$ and 200μg/ml $Ca(NO_3)_2.4H_2O$ (Galletta, 1983). In each 1.5 ml Eppendorf approximately 1000 pollen grains were suspended by the edge of a needle into 100 microliters of growth medium. RNase was suspended in the pollen growth medium to a final concentration of 0.05, 0.5, 5, 50 or 500 units/ml. The percentage of germination was examined after two hours

of incubation at 25°C in the dark and the length of the pollen-tubes was measured after incubation overnight at the same conditions. Table 1 shows that the RNase produced by A. niger has a clear inhibitory effect on both pollen germination (P<0.02) and pollen tube growth (P<0.01). For a comparison, the results obtained from the same conditions with 5 units/ml bovine pancreatic RNase are also demonstrated.

EXAMPLE 5:

Field Experiments

The RNase produced by A. niger was examined in the field as a thinning agent of deciduous fruit trees. In peach (Prunus persica) and plum (Prunus salicina) the fungal RNase was applied exogenously directly on the stigmas, by spraying the flowers during anthesis.

The flowers were exposed to open pollination and the fruit-set was examined four weeks after the blooming period. In trees of peach cv. Texas grown in an orchard in Kefar Varburg different branches were treated as follows:

- The flowers were left untreated (control)
- The flowers were sprayed by 10 mM citrate buffer pH 3.5 just once, when the percentage of open flowers was estimated as at least 50% (B-I)

- The same buffer was used, only spraying procedure was repeated every 2-3days, starting from 10% open flowers till the branch ceased blooming (B-M).

The flowers were sprayed by RNase that was diluted by the above buffer to 1 or 100 units/ml. RNase was applied once (R1-I and R100-I, respectively) or repeatedly (R1-M and R100-M respectively) during anthesis.

Table 2 shows that RNase caused a significant reduction of about 40% ($P<0.01$) of fruit-set. The repeated treatments showed better results than one treatment but interestingly, the low concentration was most effective in this case.

TABLE 1

POLLEN GERMINATION AND POLLEN TUBE LENGTH
IN DIFFERENT CONCENTRATIONS OF RNASE

RNase Conc. (units/mililiter	Number of Repetitions	Pollen Germination %	Pollen tube length (mm)
0 (control)	3	77.22±2.98	
	20		0.57±0.10
0.5	2	81.05±8.95	
	10		0.56±0.14
5	2	50.63±0.62	
	17		0.33±0.08
5[a]	5	42.89±2.97	
	55		0.31±0.08

a = pancreatic RNase.

TABLE 2.

FRUIT SET OF PEACH CV TEXAS IN KEFAR VARBURG

Treatment	Number of branches	Total number of flowers	Percentage of fruits per branch	P=0.05
Control	7	166	98.45 ±2.83	a
B-I	8	188	88.91 ±12.36	ab
B-M	9	186	84.08 ±7.54	b
R1-I	8	214	78.95 ±15.32	bc
R1-M	8	202	61.51 ±20.97	d
R100-I	7	160	66.85 ±15.42	cd
R100-M	7	217	75.32 ±9.91	bcd

In nectarine cv. Fantasia in Rosh-Zurim the effect of the pH of the solvent and the effect of Triton x-100 as a surfactant were examined. All the branches were sprayed repeatedly, starting from 10% blooming. The treatment were designed as follows:

- Untreated flowers as control.

- Flowers that were sprayed by 100 units/ml of RNase that was dissolved in water or 10mM citrate buffer pH 3.5 (RW and RB, respectively), without or with 0.025% Triton X-100 (RWT and RBT, respectively). Table 3 shows that RNase significantly reduced fruit set in nectarine trees. The use of a buffer in addition to Triton X-100 did not significantly alter the results.

In plum cv. Vikson branches were sprayed with 1, 10 or 100 units/ml of RNase, dissolved in water (R1, R10 and R100, respectively, supplemented with 0.025% Triton X-100 and applied once at 50% blooming (I) or three times, starting from 10% blooming (M). Table 4 shows that the combination of high concentration of RNase and repeated applications during anthesis were most effective in reducing fruit-set.

TABLE 3

FRUIT SET OF NECTARINE CV. FANTASIA IN ROSH ZURIM

Treatment	Number of branches	Total number of flowers	Percentage of fruits per branch	P=0.05
Control	5	100	63.60 ±13.64	a
RW	5	81	41.80 ±8.70	b
RWT	5	89	43.75 ±7.74	b
RB	5	97	45.0 ±9.56	b
RBT	5	85	41.0 ±14.16	ib

R = Spraying with RNase (extract from *Aspergillus niger*)
W = Water as Solvent
B = Buffer as a solvent
T = Triton x 100

TABLE 4

FRUIT SET OF PLUM CV VIKSON IN HELETZ[a]

No.	Treatment	Concentration of RNase Per ml	Percent of Fruit Set In Experiment A[b]		Experiment B[c]	
1	Control		6.2	ab	13.0	a
2	RNase	1	9.0	a	9.7	ab
3	RNase	10	5.7	ab	7.8	ab
4	RNase	100	3.4	b	5.5	b

a = At a certainty of P=0.05

b Experiment A = Three sprayings starting at 10% flowering

c Experiment B = Single spraying at 50% flowering.

EXAMPLE 6

Effect on Shotberries in Grape-Vines

The vineyard tested did not receive any sort of spraying of gibberellin to increase the size of the grapes. The vine was sprayed by hand during flowering on 6/5/93. On 11/7/93 the bunches were weighed and the number of shot berries counted. The results are shown in Table 5.

EXAMPLE 7

RNase samples from the fermentation of Aspergillus niger were loaded on 15% polyacrylamide gel electrophoresis (SDS PAGE). The proteins were resolved and after the run the proteins were renatured in situ by removing the SDS using an isopropanol buffer. After the run the gel was divided into two lanes. One lane was subjected to RNase zymogram detection (overlayed on 0.1% yeast RNA and 1.5% agarose for several hours) and then stained with 0.1% toluidine blue. RNase bands were visualized as evident by a white halo around an active band at approximately 15KDa and 30 KDa. The corresponding band from the parallel lane was dissected and inserted into peach pollen germinating tubes. The percent of germination and pollen tube growth were recorded after 24 hours. The results (pollen tube length) are shown in Table 6.

TABLE 5

THE EFFECT OF RNASE ON SHOTBERRIES IN GRAPES[a]

No.	Treatment	Con. RNase	No.Shotberries Per Bunch	No. Shotberries Per 100g Grapes
1	control	-	77.1 a	45.1 a
2	RNase	1u/ml	84.5 a	46.9 a
3	RNase	10u/ml	45.5 b	33.8 ab
4	RNase	100u/ml	38.9 b	18.6 b

a = At a certainty of p=0.05

TABLE 6

POLLEN TUBE LENGTH OF RNASE SAMPLES ISOLATED FROM THE FERMENTATION OF A. NIGER

	Untreated	Gel Without Proteins	Bands at 30 Dg
Average	0.48 a	0.664 b	0.33 c
Standard	0.19	0.38	0.24
STE[a]	0.03	0.06	0.03

a = At p = 0.05

EXAMPLE 8

The effect on the germination and the pollen tube length of peach pollen with boiled RNase (originating from Aspergillus niger) was studied. The results shown in Tables 7 and 8 show a marked effect even after boiling the RNase.

TABLE 7

PEACH POLLEN GERMINATION IN BOILED RNASE[a]

Percent Germination	Rnase Concentration (unit/ml
45.0 a	0
40.5 ab	0.09
30.4 b	0.9
0	9

a = From Aspergillus niger

TABLE 8

PEACH POLLEN TUBE LENGTH IN BOILED RNASE[a]

Pollen Tube Length (mm)	Rnase Concentrations (units/ml)
0.55 a	0
0.48 b	0.09
0.30 c	0.9

a = from Aspergillus niger.

CLAIMS

1. A method of the biological control of pollen in plants applying an affective amount of activated or inactivated RNase or a mixture of these.

2. A method in accordance with Claim 1, wherein the inactivated RNase is chosen from the group consisting of protein, protein fragments, ribozymes, polypeptides and mixture of these.

3. A method in accordance with Claim 1 or 2, wherein the RNase is obtained from sources chosen from the group consisting of bacterial, plant, animal, fungal and viral.

4. A method in accordance with any of Claims 1 to 3, wherein the RNase is secreted by fungal sources chosen from the group consisting of Aspergillus niger, Aspergillus clavetur, Aspergillus oryzae and Rhisopus miveus.

5. A method in accordance with Claim 4, wherein the fungal sources are chosen from the group consisting of Aspergillus niger B1 and Aspergillus oryzae.

6. A method in accordance with any of Claims 1 to 5, wherein the plants are chosen from the group consisting of fruit trees, ornamental trees, vegetables field crops, plantations and ornamentals.

7. A method in accordance with any of Claims 1 to 6, wherein the trees are chosen from the group consisting of deciduous, tropical, subtropical, citrus, nut trees and grape vines.

8. A method in accordance with Claim 7, wherein the deciduous plants are chosen from the group consisting of stone fruit, pears, apples, quince, peaches, plum, nectarines, cherries, and apricots.

9. A method in accordance with Claim 7, wherein the subtropical plants are chosen from the group consisting of avocado, mango, and persimmon.

10. A method in accordance with Claim 7, wherein the citrus plants are chosen from the group consisting of orange, lemon, grapefruit, clementine, mandarin, citrus, pomela and their hybrids.

11. A method in accordance with Claim 6, wherein the field crops are cereals.

12. A method in accordance with Claim 6, wherein the ornamental trees are chosen from the group consisting of olive trees and ficus sp.

13. A method of preventing the self- or cross-fertilizations of plants by applying an effective amount of activated or inactivated RNase or a mixture of these.

14. A method in accordance with Claim 13, wherein the inactivated RNase is chosen from the group consisting of protein, protein fragments, ribozymes, polypeptides, and mixture of these.

15. A method in accordance with Claim 13 or 14, wherein the RNase is obtained from sources chosen from the group consisting of bacterial, plant, animal, fungal and viral.

16. A method in accordance with any of Claims 13 to 15, wherein the RNase is secreted by fungal sources chosen from the group consisting of Aspergillus niger, Aspergillus clavetur, Aspergillus oryzae, and Rhisopus miveus.

17. A method in accordance with Claim 16, wherein the fungal sources are chosen from the group consisting of Aspergillus niger B1 and Aspergillus oryzae.

18. A method in accordance with any of Claims 13 to 17, wherein the plants are chosen from the group consisting of fruit trees, ornamental trees, vegetables field crops, plantations and ornamentals.

19. A method in accordance with any of Claims 13 to 18, wherein the trees are chosen from the group consisting of deciduous, tropical, subtropical, citrus, nut trees, and grape vines.

20. A method in accordance with Claim 19, wherein the deciduous plants are chosen from the group consisting of stone fruit, pears, apples, quince, peaches, plum, nectarines, cherries, and apricots.

21. A method in accordance with Claim 19, wherein the subtropical plants are chosen from the group consisting of avocado, mango and persimmon.

22. A method in accordance with Claim 19, wherein the citrus plants are chosen from the group consisting of orange, lemon, grapefruit, clementine, mandarin, citrus, pomela and their hybrids.

23. A method in accordance with Claim 18, wherein the field crops are cereals.

24. A method in accordance with Claim 18, wherein the ornamental trees are chosen from the group consisting of olive trees and ficus sp.

25. A method of thinning fruit by applying an effective amount of activated or inactivated RNase or a mixture of these to the fruit trees during anthesis.

26. A method in accordance with Claim 25, wherein the inactivated RNase is chosen from the group consisting of protein, protein segments, ribozymes, polypeptides, and mixture of these.

27. A method in accordance with Claim 25 or 26, wherein the RNase is obtained from sources chosen from the group consisting of bacterial, plant, animal, fungal and viral.

28. A method in accordance with any of Claims 25 to 27, wherein the RNAase is secreted by fungal sources chosen from the group consisting of Aspergillus oryzae and Rhisopus miveus.

29. A method in accordance with Claim 28, wherein the fungal sources are chosen from the group consisting of Aspergillus niger B1 and Aspergillus oryzae.

30. A method in accordance with any of Claims 25 to 29, wherein the plants are chosen from the group consisting of fruit trees, ornamental trees, vegetables field crops, plantations and ornamentals.

31. A method in accordance with any of Claims 25 to 30, wherein the plants are chosen from the group consisting of deciduous, tropical, subtropical, citrus, nut trees, and grape vines.

32. A method in accordance with Claim 31, wherein the deciduous plants are chosen from the group consisting of stone fruit, pears, apples, quince, peaches, plum, nectarines, cherries and apricots.

33. A method in accordance with Claim 31, wherein the subtropical plants are chosen from the group consisting of avocado, mango and persimmon.

34. A method in accordance with Claim 31, wherein the citrus plants are chosen from the group consisting of orange, lemon, grapefruit, clementine, mandarin, citrus, pomelo and their hybrids.

35. A method of reducing seeds in plants by applying an effective amount of activated or inactivated RNAse or a mixture of these to the plants during anthesis.

36. A method in accordance with Claim 35, wherein the inactivated RMAse is chosen from the group consisting of protein, protein segments, ribozymes, polypeptides, and mixture of these.

37. A method in accordance with Claims 35 or 36, wherein the RNAse is obtained from sources chosen from the group consisting of bacterial, plant, animal, fungal and viral.

38. A method in accordance with any of Claims 35 to 37, wherein the RNAse is secreted by fungal sources chosen from the group consisting of Aspergillus niger, Aspergillus clavetur, Aspergillus oryzae and Rhisopus miveus.

39. A method in accordance with Claim 38, wherein the fungal sources are chosen from this group consisting of Aspergillus niger B1 and Aspergillus oryzae.

40. A method in accordance with any of Claims 35 to 39, wherein the plants are chosen from the group consisting of citrus, subtropicals and grapes.

41. A method in accordance with Claim 40, wherein the citrus plants are chosen from the group consisting of orange, lemon, grapefruit, clementine, mandarin, citron, pomela and their hybrids.

42. A method in accordance with Claim 40, wherein the subtropical plants are chosen from the group consisting of avocado, mango and persimmon.

43 A method of reducing the incidence of shotberries in grape vines by applying an effective amount of activated or inactivated RNase or a mixture of these to the grape vines.

44. A method in accordance with Claim 43, wherein the inactivated RNAse is chosen from the group consisting of protein, protein fragments, ribozymes, polypeptides and a mixture of these.

45. A method in accordance with Claim 43 or 44, wherein the RNAse is obtained from sources chosen from the group consisting of bacterial, plant, animal, fungal and viral.

46. A method in accordance with any of Claims 43 to 45, wherein the RNAse is secreted by fungal sources chosen from the group consisting of Aspergillus niger, Aspergillus clavetur, Aspergillus oryzae and Rhisopum miveus.

47. A method in accordance with Claim 46, wherein the fungal sources are chosen from the group consisting of Aspergillus niger B1 and Aspergillus oryzae.

48. A method applying an effective amount of the RNAse to plants via bees.

49. A method in accordance with any of Claims 1 to 48 with reference to the examples.

INTERNATIONAL SEARCH REPORT

International application No. PCT/US94/10082

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/54, 38/44
US CL :424/94.1, 94.2, 94.21, 94.4
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.1, 94.2, 94.21, 94.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, APS, BIOSIS, DIALOG
search terms: (ribonuclease or rnase) and pollen

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Plant Cell, Volume 1, issued May 1989, W. Jahnen et al., "Inhibition of in Vitro Pollen Tube Growth by Isolated S-Glycoproteins of *Nicotiana alata*", pages 501-510, see entire document.	1-49
Y	Nature, Volume 342, issued 21 December 1989, B.A. McClure et al., "Style Self-incompatibility Gene Products of *Nicotiana alata* are Ribonucleases", pages 955-957, see entire document.	1-49
Y	Nature, Volume 347, issued 25 October 1990, B.A. McClure et al., "Self-incompatibility in *Nicotiana alata* Involves Degradation of Pollen rRNA", pages 757-760, see entire document.	1-49

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 NOVEMBER 1994	30 JAN 1995

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	JEAN C. WITZ
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196