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(54) Title: IMPROVING THE YIELD OF PLANTS			
(57) Abstract <p>The invention relates to the exogenous use of betaine to improve the yield of grain legumes. According to the invention, betaine is used to improve the yield especially under stress conditions. The invention also relates to grain legumes treated exogenously with betaine, and particularly to the seeds of such plants.</p>			

Improving the yield of plants**Technical field**

5 The invention relates to the use of betaine to
improve the yield of plants. The invention relates
especially to the use of betaine to improve the yield
of grain legumes. According to the invention, the yield
10 can be improved both under normal and stress conditions,
i.e. when the conditions are poor due to e.g. drought,
high salinity, low temperatures, humidity or
environmental pollutants interfering with the growth.
The invention also relates to grain legumes treated with
betaine and to the parts thereof, especially seeds, and
15 to products prepared from these.

Background

 The environment and conditions for growth
considerably affect the yield of plants. Optimum growth
environment and conditions usually result in a yield
20 that is large in quantity and high in quality. Under
poor growth conditions both the quality and the quantity
naturally deteriorate.

 The physiological properties of a plant are
preferably manipulated by means of breeding, both with
25 traditional breeding methods and for example with
genetic manipulation.

 Several different solutions concerning
cultivation technique have been developed to improve the
growth conditions and yield of plants. Selecting the
30 right plant for the right growth location is self-
evident for a person skilled in the art. During the
growing season plants may be protected with mechanical
means by utilizing for example different gauzes or
plastics or by cultivating the plants in greenhouses.
35 Irrigation and sprinkler irrigation, fertilizers and

plant nutrients are generally used in order to improve the growth. Surfactants are often used in connection with applying pesticides, protective agents and minerals. Surfactants improve the penetration of these substances into plant cells, thereby enhancing and increasing the effect of the aforementioned agents and simultaneously reducing their harmful effects on the environment. However, different methods of cultivation technique are often laborious and impractical, their effect is limited (the economical size of a greenhouse, the limited protection provided by gauzes, etc.), and they are also far too expensive on a global scale. No economically acceptable chemical solutions for protecting plants from stress conditions have been described so far.

Water supply is more important than any other environmental factor for the productivity of a crop, even though the sensitivity of plants to drought varies. Irrigation is usually utilized to ensure sufficient water supply. However, there are significant health and environmental problems related to irrigation, for example a sharp decrease in water resources, deterioration of water quality and deterioration of agricultural lands. It has been calculated in the field that about half of the artificially irrigated lands of the world are damaged by waterlogging and salinization. An indication of the significance and scope of the problem is that there are 255 million hectares of irrigated land in the world, and they account for 70% of the total world water consumption. In the United States alone, there are over 20 million hectares of irrigated land mainly in the area of the 18 western states and in the southeastern part of the country. They use 83% of the total water consumption for irrigation alone. It can also be noted that the use of irrigation

water increases every year especially in industrial countries. In addition to these problems, another drawback of irrigation is the high cost.

5 Another serious stress factor is the salinity of soil. The salinity of soil can be defined in different ways; according to the general definition, soil is saline if it contains soluble salts in an amount sufficient to interfere with the growth and yield of several cultivated plant species. The most common of the
10 salts is sodium chloride, but other salts also occur in varying combinations depending on the origin of the saline water and on the solubility of the salts.

15 It is difficult for plants growing in saline soil to obtain a sufficient amount of water from the soil having a negative osmotic potential. High concentrations of sodium and chloride ions are toxic to plants. An additional problem is the lack of minerals, which occurs when sodium ions compete with potassium ions required, however, for cell growth, osmoregulation and pH stabilization. This problem occurs especially
20 when the calcium ion concentration is low.

The productivity of plants and their sensitivity to the salinity of soil also depend on the plant species. Halophytes require relatively high sodium chloride contents to ensure optimum growth, whereas
25 glycophytes have low salt tolerance or their growth is considerably inhibited already at low salt concentrations. There are great differences even between different cultivars of a cultivated plant species. The salt tolerance of one and the same species or cultivar may also vary depending for example on the stage of
30 growth. In the case of low or moderate salinity, the slower growth of glycophytes cannot be detected in the form of specific symptoms, such as chlorosis, but it is shown in the stunted growth of the plants and in the
35

colour of their leaves that is darker than normal. Moreover, the total leaf area is reduced, carbon dioxide assimilation decreases and protein synthesis is inhibited.

5 Plants can adapt to some extent to growth and stress conditions. This ability varies considerably depending on the plant species. As a result of the
10 aforementioned stress conditions, certain plants begin to produce a growth hormone called abscisic acid (ABA), which helps the plants to close their stomata, thus
15 reducing the severity of stress. However, ABA also has harmful side effects on the productivity of plants. ABA causes for example leaf, flower and young fruit drop and inhibits the formation of new leaves, which naturally
20 leads to reduction in yield.

 Stress conditions and especially lack of water have also been found to lead to a sharp decrease in the activity of certain enzymes, such as nitrate reductase and phenylalanine ammonium lyase. On the other hand, the
25 activity of alpha-amylase and ribonuclease increases. No chemical solutions, based on these findings, to protect plants have been described so far.

 It has also been found that under stress conditions certain nitrogen compounds and amino acids,
25 such as proline and betaine, are accumulated in the regions of growth of certain plants. The literature of the art discusses the function and meaning of these accumulated products. On the one hand it has been
30 proposed that the products are by-products of stress and thus harmful to the cells, on the other hand it has been estimated that they may protect the cells (Wyn Jones, R.G. and Storey, R.: *The Physiology and Biochemistry of Drought Resistance in Plants*, Paleg, L.G. and Aspinall, D. (Eds.), Academic Press, Sydney, Australia, 1981).

Zhao et al. (in *J. Plant Physiol.* 140 (1992) 541 - 543) describe the effect of betaine on the cell membranes of alfalfa. Alfalfa seedlings were sprayed with 0.2M glycinebetaine, whereafter the seedlings were uprooted from the substrate, washed free of soil and exposed to temperatures from -10°C to -2°C for one hour. The seedlings were then thawed and planted in moist sand for one week at which time regrowth was apparent on those plants that had survived. Glycinebetaine clearly improved the cold stability of alfalfa. The effect was particularly apparent at -6°C for the cold treatment. All controls held at -6°C for one hour died, whereas 67% of the seedlings treated with glycinebetaine survived.

Itai and Paleg (in *Plant Science Letters* 25 (1982) 329 - 335) describe the effect of proline and betaine on the recovery of water-stressed barley and cucumber. The plants were grown in washed sand, and polyethylene glycol (PEG, 4000 mol. wt.) was added to the nutrient solution for four days in order to produce water stress, whereafter the plants were allowed to recover for four days before harvesting. Proline and/or betaine (25mM, p 6.2) was sprayed on the leaves of the plant either on the first or third day of the stress or immediately before harvesting. As regards barley, it was noted that betaine supplied either before or after the stress had no effect, whereas betaine added in the end of the stress was effective. Proline had no effect. No positive effect was apparent for cucumber. On the contrary, it was found out that both betaine and proline had a negative effect.

Experiments aiming at clarifying the effects of betaine and proline on plants have thus yielded contradictory results. There are no commercial applications based on these results.



Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", means "including but not limited to" and is not intended to
5 exclude other additives, components, integers or steps.

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Brief description of the invention

The purpose of the present invention was to find a way to partially replace irrigation so that the amount and quality of the yield could be simultaneously
5 ensured. Another purpose of the invention was to find a way to protect plants also under other stress conditions, such as during high salinity often connected with drought, at low temperatures, etc. Moreover, a further aim was to find a way to increase the yield
10 under normal conditions without utilizing methods that would consume environmental resources or harm the environment.

In connection with the present invention it has been proved that the yield of grain legumes can be
15 considerably improved by means of exogenously applied betaine. Betaine has been found to be effective in improving the yield both under normal and stress conditions, and it has no such detrimental effects as the side effects of ABA. Betaine application makes it
20 possible to considerably reduce for example the need for artificial irrigation, thus saving the environment and cutting down the costs to a great extent. An advantageous feature of the invention is also the decrease of the antinutrient concentration of plants as
25 a result of the betaine application. A good example of this is the low alkaloid content of lupins treated with betaine, i.e. about half of the normal level.

The invention thus relates to the exogenous use of betaine to improve the yield of grain legumes. The
30 invention relates especially to the use of betaine to improve the seed yield of grain legumes.

According to the invention, betaine is used exogenously to improve the yield of grain legumes both under normal and stress conditions.

The invention also relates to the exogenous use of betaine to reduce the antinutrient content of grain legumes, especially to reduce the alkaloid content of lupin.

5 The invention further relates to grain legumes treated exogenously with betaine and to the parts thereof, particularly seeds, and to their use as such and for example in food, animal feed and forage industries.

10 The invention also relates to a method of improving the yield of grain legumes, in which method betaine is exogenously applied to growing grain legumes.

15 The invention further relates to a method of reducing the antinutrient content of grain legumes, in which method betaine is exogenously applied to growing grain legumes. The invention especially relates to a method of reducing the alkaloid content of lupins, in which method betaine is exogenously applied to growing lupins.

20 Betaine is applied to the plant in either one or several dosages. The application may be performed for example by spraying together with some other spraying of for example a pesticide, if desired. Betaine used according to the invention is transported to plant cells, where it actively regulates the osmotic balance of the cells and also participates in other processes of cell metabolism. A plant cell treated with betaine is more viable even when subjected to exogenous stress factors.

25 The betaine treatment according to the invention is economically advantageous, and the yield increases in an amount that is economically profitable and significant. The treatment does not produce significantly more work since it may be performed together with other sprayings, and it does not require

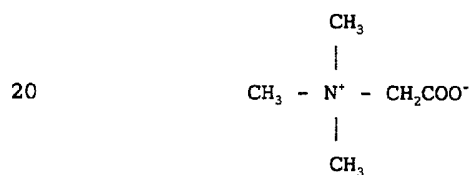
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new investments in machinery, equipment or space. It should also be noted that betaine is a non-toxic natural product, which has no detrimental effects on the quality of the yield. Betaine is also a stable substance that
5 remains in the plant cells and thereby has a long-standing effect.

Detailed description of the invention

Betaine refers to fully N-methylated amino acids. Betaines are natural products that have an
10 important function in the metabolism of both plants and animals. One of the most common betaines is a glycine derivative wherein three methyl groups are attached to the nitrogen atom of the glycine molecule. This betaine compound is usually called betaine, glycinebetaine or
15 trimethylglycine, and its structural formula is presented below:



Other betaines are for example alaninebetaine
25 and prolinebetaine, which has been reported to for example prevent perosis in chicks. R.G. Wyn Jones and R. Storey describe betaines in detail in *The Physiology and Biochemistry of Drought Resistance in Plants* (Paleg, L.G. and Aspinall, D. (Eds.), Academic Press, Sydney,
30 Australia, 1981). The publication is included herein by reference.

Betaine has a bipolar structure and it contains several chemically reactive methyl groups which it can donate in enzyme-catalyzed reactions. Most organisms can
35 synthesize small amounts of betaine for example for the

methyl function, but they cannot react to stress by substantially increasing the production and storage of betaine. Best known organisms accumulating betaine are plants belonging to the *Chenopodiaceae* family, for example sugar beet, and some microbes and marine invertebrates. The main reason for the betaine accumulation in these organisms is probably that betaine acts as an osmolyte and thus protects the cells from the effects of osmotic stress. One of the main functions of betaine in these plants and microbes is to increase the osmotic strength of the cells when the conditions require this, for example in case of high salinity or drought, thus preventing water loss. Unlike many salts, betaine is highly compatible with enzymes, and the betaine content in cells and cell organelles may therefore be high without having any detrimental effect on the metabolism. Betaine has also been found to have a stabilizing effect on the operation of macromolecules; it improves the heat resistance and ionic tolerance of enzymes and cell membranes.

Betaine can be recovered for example from sugar beet with chromatographic methods. Betaine is commercially available from Cultor Oy, Finnsugar Bioproducts as a product that is crystalline water-free betaine. Other betaine products, such as betaine monohydrate, betaine hydrochloride and raw betaine-containing liquids, are also commercially available and they can be used for the purposes of the present invention.

According to the present invention, betaine is thus used exogenously to improve the yield of grain legumes, such as soybean, faba bean, green bean and other beans, pea, lupin, etc. According to the invention, betaine is used to improve the yield of grain legumes both under normal and stress conditions, i.e.

when the plants are subjected to periodic or continuous exogenous stress. Such exogenous stress factors include for example drought, high temperatures, high soil salinity, air pollution, such as ozone, nitrogen oxides, sulphur dioxide and sulphuric acid (acid rain), environmental poisons, herbicides, pesticides, etc. Treating plants subjected to stress conditions exogenously with betaine for example improves the adaptation of the plants to the conditions and maintains their growth potential longer, thereby improving the yield-producing capacity of the plants. Betaine is also a stable substance that remains in the plant cells. The positive effect of betaine is thereby long-standing and diminishes only gradually due to dilution caused by the growth.

Even though this reference and the claims use the word 'betaine', it is clear that according to the invention several different betaines can be used, if desired. It should also be noted that betaine is used here as a general term which thus covers different known betaines.

Betaine is applied to the plants in either one or several dosages. Application in a single dose is considered preferable. The amount used varies depending on the grain legume species and cultivar, and on the stage and conditions of growth. A useful amount may be for example about 0.1 to 20 kg of betaine per hectare. A preferable amount is thus for example about 1 to 6 kg of betaine per hectare. The amounts given here are only suggestive; the scope of the present invention thus contains all amounts that work in the manner described herein.

Any method suitable for the purpose may be used for the application of betaine. Betaine can be applied separately or together with other plant protectants,

pesticides or nutrients, such as fungicides and urea or micronutrients. Betaine can be applied easily for example by spraying. Foliar application of betaine and possible other agents through spraying is a preferable method which enables a more rapid response than methods involving root application. However, there may be different problems related to this method, such as low penetration concentrations in leaves with thick cuticles, run-off from hydrophobic surfaces, washing off by rain, rapid drying of the solution and leaf damage, and therefore other methods may also be used to apply betaine, if desired.

According to the invention, an aqueous solution of betaine is preferably used.

The time of the treatment according to the invention may also vary. If betaine is applied in a single dosage, the treatment is usually performed at an early stage of growth, for example on plants of about 5 to 20 cm, or when the leaves have just come out. If betaine is applied in several dosages, a new spraying is performed preferably in the beginning of flowering or when stress can be forecasted on the basis of the weather.

The betaine treatment according to the invention considerably improves the yield of grain legumes, for example the amount and quality of the yield. The treatment according to the invention can also reduce the need for artificial irrigation. The treatment according to the invention is economically advantageous and the increase in yield is economically profitable and significant. In connection with the invention it has been shown that for example lupin yield can be increased by over 28% with a suitable betaine dosage, for example about 6 kg/ha. It should also be noted that even though the amount of yield increases to a considerable extent,

the quality does not deteriorate. On the contrary, in connection with the present invention it has been shown that the antinutrient content of plants, for example the alkaloid content of lupins, considerably decreases as a result of the betaine application according to the invention.

High concentrations of alkaloids are poisonous to animal cells, and therefore a low alkaloid content in lupins is an important criterion of quality in view of lupin applications. One of the quality requirements for use in foodstuffs is that lupin seeds contain less than 0.02% of alkaloids. There has generally been a tendency to maintain the level of alkaloids as low as possible by selecting cultivars with a low alkaloid content. Since lupin cultivars with higher alkaloid contents produce higher yields, this approach has not been considered very advantageous. Eliminating alkaloids or reducing their amount during the processing of lupins has also been utilized, but this naturally increases the number of processing steps and the costs.

Most of the lupin yield is used as forage or in some other form as food for animals whereupon the advantages of lupin are its high contents of protein, amino acids and energy. The highest allowed alkaloid content for these applications is 0.04%. However, even with low concentrations alkaloids cause a bitter off-taste, wherefore animals tend to avoid eating forage or other feed containing alkaloids. The use of lupins in animal food and forage applications is therefore restricted due to their alkaloid content. It is also known that the alkaloid content of lupins increases under stress conditions.

The considerable decrease in the alkaloid content of lupins achieved with the betaine treatment according to the present invention is therefore a

significant additional advantage which is even more marked in view of the notable positive effect of betaine on the stress resistance of plants.

5 According to the invention, the yield of grain legumes can thus be improved both under normal and stress conditions, which in addition to drought include for example high salinity often connected with drought, high temperature, etc. Furthermore, the invention also makes it possible to grow grain legumes on lands that
10 were previously considered unfit for cultivation.

The invention will be described in greater detail by means of the following examples. The examples are only provided to illustrate the invention, and they should not be considered to limit the scope of the
15 invention in any way.

Example 1

Effect of betaine application on lupin yield

The effect of betaine application on lupin yield was examined at Murdoch University, Perth, Australia. The experiment was conducted under field
20 conditions during the winter of 1994, which was cooler and rainier than usual, but during which water stress did occur, however.

The experiment was conducted according to a
25 split-plot design utilizing plots of 8 m². The plots were divided into four sub-plots that were treated with different betaine concentrations. The betaine concentrations used were 0 (control), 2 kg/ha, 4 kg/ha and 6 kg/ha. The soil was sandy (98% sand, 1% silt and
30 1% clay) with a low nitrogen, phosphorus and potassium content and poor water and nutrient retention properties. The amount of irrigation was normal. The cultivar was Gungurru. The results are shown in Table 1.

Table 1
Effect of betaine application on lupin yield

betaine concentration (kg/ha)	yield of lupin seeds	
	kg	% of control
0 (control)	0.979	100
2	0.992	101
4	1.048	107
6	1.253	128

The results show that the yield increased over the control in all the experiments conducted. The best results were obtained with a betaine application rate of 4 or 6 kg/ha.

Example 2

Effect of betaine application on lupin yield under dry conditions

The effect of betaine application on lupins growing under water stress was examined by repeating the experiment described in Example 1, but with a 50% reduction in irrigation from the optimum amount. The results are shown in Table 2.

Table 2
Effect of betaine application on lupin yield under water stress

betaine concentration (kg/ha)	yield of lupin seeds	
	kg	% of control
0 (control)	0.856	100
2	0.938	110
4	0.912	107
6	1.031	120

The yield also increased clearly in this experiment compared with the control. It can also be noted that utilizing the higher glycine betaine concentration of 6 kg/ha provided similar results with a low irrigation level (50%) as utilizing the lower betaine rate of 2 to 4 kg/ha with optimum irrigation (Example 1). This means that the same yield can be achieved by decreasing irrigation if a higher betaine application rate is used simultaneously.

10 Example 3

Effect of betaine application on the alkaloid content of lupins

The alkaloid content of lupins grown and treated according to Examples 1 and 2 was determined with the method described by Priddis [Journal of Chromatography, 261 (1983) 95 - 101]. The results are shown in Table 3.

Table 3

Effect of betaine application on the alkaloid content of lupin seeds

treatment	alkaloid content (%)
control I irrigation rate 50%	0.04
6 kg/ha betaine irrigation rate 50%	0.02
control II irrigation rate 100%	0.02
6 kg/ha betaine irrigation rate 100%	0.01

The results show a clear decrease in the total alkaloid content of lupins, which is a very surprising and positive result from the betaine application according to the invention.

Example 4**Effect of betaine application on the early development of green bean seeds**

5 The effect of betaine on the germination frequency and rate of green bean seeds was examined using water as control. The green bean was of the type Spartan Arrow Bush Bean Lot #1987-3, produced by Northrup King Co. Three different test solutions were prepared for the experiments as follows:

10

Test solution	pH
A deionized water	7.01
B betaine (0.02 g/l)	6.34
C betaine (2 g/l)	6.80

15

Twenty green bean seeds were soaked for 24 hours in 330 ml of one of the aforementioned test solutions. The seeds were then dried on stainless steel screens and sown into soil with two seeds placed in each container. 20 The containers were then placed on a window ledge with a southern exposure to the sun, and they were watered daily with deionized water.

The early development of the seeds was followed by determining both the rate and frequency of 25 germination. The first measurements were performed ten days after the experiment began, and the second set of measurements was conducted 19 days after the beginning of the experiment. The results are shown in Table 4.

Table 4

Effect of betaine application on the early development of green bean seeds

Betaine concentration (g/l)	Germination frequency (%)		Average shoot length	
	Day 10	Day 19	(inch)	(% of control)
0 (control)	0	40	2.25	100
0.02	80	100	4.82	214
2	20	100	3.88	172

10

The results show that betaine promotes faster germination in green beans. Betaine also produced changes in the look of plants, for example the colour of leaves became dark green. The best results were achieved with the lower betaine rate of 0.02 g/l.

15

Example 5

Effect of betaine application on pea yield under stress conditions

20

The effect of betaine application on the yield of peas growing under stress conditions was examined in the following way. Peas were sown in 5 l plastic pots containing a mixture of peat and vermiculite in a ratio of 1:1. The plants were grown in greenhouses at a mean day/night temperature of 28°C/12°C and a relative humidity of between 42 and 45%. Supplemental illumination was provided for 17 hours a day with tungsten lamps (PAR 434 $\mu\text{mol m}^{-2}\text{s}^{-1}$). Twenty seeds were sown per pot and they were later thinned to 10 plants per pot. The total number of pots used was twenty-four, twelve of which were exposed to drought, i.e. water stress, and twelve to high salinity, i.e. salt stress.

25

30

A completely randomised design was utilized in the experiment, with 4 replications.

5 Water stress (pF3) was imposed on half of the plants four weeks after seedling emergence. The pots were then grouped into 3 sets, each of which consisted of 4 pots, and each set was sprayed with either 25 ml of distilled water, 0.1M betaine solution or 0.3M betaine solution two weeks after the stress imposition.

10 To induce salt stress, 200 ml of 100 mM NaCl solution was applied to half of the pots every four days for five weeks after seedling emergence. The pots were grouped in 3 sets of 4 pots in each set, and they were sprayed with 25 ml of distilled water, 0.1M betaine solution or 0.3M betaine solution after the first administration of the NaCl solution. The NaCl treatment was repeated six more times after the betaine application.

15 At harvest, the total number of nodules, number of active nodules, number of pods, and leaf dry matter content were determined. The results are shown in Tables 5 and 6.

Table 5
Effect of betaine application on the nodulation and growth of pea under water stress

betaine concentration (M)	number of active nodules		number of pods		leaf dry matter content	
		% of control		% of control	g	% of control
0	0.68	100	1.55	100	0.21	100
0.1	0.77	113	1.84	119	0.26	124
0.3	0.63	93	1.46	94	0.18	85

Table 6
Effect of betaine application on the nodulation and growth of pea under salt stress

betaine concentration (M)	number of nodules		number of active nodules		number of pods		leaf dry matter content	
		% of control		% of control		% of control	g	% of control
0	8.20	100	0.36	100	2.08	100	0.27	100
0.1	15.74	192	1.58	439	2.20	106	0.33	122
0.3	13.16	160	0.33	92	1.69	81	0.29	107

The smaller betaine rate of 0.1M thus had a positive effect both on the number of active nodules, number of pods and the leaf dry matter content when the pea was growing under dry conditions. The positive effect on pea
5 growing under salt stress was even clearer. The higher betaine content of 0.3M had a positive effect on the number of nodules and leaf dry matter content of peas growing under salt stress.

Example 6

10 **Effect of betaine application on the growth rate of pea**

The experiment of Example 5 was repeated by utilizing betaine solutions of 0 (control), 0.05M, 0.1M and 0.2M. Water stress was induced in the manner
15 described in Example 5, whereas salt stress was not examined in this experiment. In order to examine the recovery of plants, the stressed plants were divided on day 28 of the experiment into two groups one of which still remained under water stress, and the other one was
20 irrigated and its recovery was followed. Samples were taken on days 21, 28, 35 and 42. Peas growing under optimum conditions (sufficient irrigation) were used as control. The best results were obtained with the betaine rate of 0.05M. The results concerning the relative
25 growth rate of pea and the dry weight of the shoot are shown in Figures 1 and 2, respectively.

Example 7

30 **Effect of betaine application on faba bean yield under stress conditions**

The experiments described in Example 5 were repeated utilizing faba bean. Ten seeds of faba bean were sown per pot and they were later thinned to 3 plants per pot. The other parameters of the experiments corresponded to those described in Example 5.

The positive effect on faba bean was apparent especially for the number of pods, which increased under water stress from a control value of 3.13 to 3.50 with the 0.1M betaine solution, and to 3.63 with the 0.3M betaine solution. The results correspond to values 112 and 116 in percentages of the control (100). The leaf dry matter content increased from a control value of 2.04 g to 2.21 g with the 0.1M betaine solution, but decreased to 1.67 g with the 0.3M betaine solution.

10 **Example 8**

Effect of betaine application on soybean yield

The effect of betaine application on soybean yield under normal and dry conditions was investigated in field conditions on a farm where the soil is very light and sandy and can retain about 35 mm of rain for a few days, but where water stress will occur in a few days even after a heavy rain. There were overhead irrigation facilities on the farm to guarantee sufficient irrigation of the control areas. In addition to the growth of soybean, the experiment also determined its nitrogen fixation capacity.

The experiments were conducted with a 3-Factor Randomized Complete Block Design with watering level (main factor), cultivar (subplot) and betaine concentration (split) as factors. The cultivars were Biloxi and Cook, which have a different drought tolerance; cultivar Cook has a more drought tolerant symbiosis system than cultivar Biloxi. The betaine levels applied included 0 (control), 3 kg/ha, and 6 kg/ha. Betaine was applied by spraying. The season had an early drought period followed by very heavy rains and again a drought period. The betaine application was repeated after the rains before the second drought period with the same application levels. The results concerning the leaf dry weight are shown in Table 7.

Table 7
 Effect of betaine application on the leaf dry weight of soybean

betaine concentration (kg/ha)	leaf dry weight (g/plant)									
	Cook					Biloxi				
	normal	% of control	dry stress	% of control	normal	% of control	dry stress	% of control	dry stress	% of control
0	13.5	100	11.1	100	15.1	100	10.5	100	10.5	100
3	14.1	104	12.6	113	16.3	108	11.5	110	11.5	110
6	17.7	131	10.2	92	15.5	103	10.6	101	10.6	101

The nitrogen fixation capacity of soybean was determined by measuring the nitrogenase activity with an acetylene reduction test wherein acetylene is reduced to ethylene. The experiment was conducted 10 weeks after sowing. In order to perform the measurement in the field, metal cylinders of 10 cm in diameter and 20 cm in depth were placed in the soil around a soybean plant. The plant was removed from the soil in the cylinder and the shoot was cut off. The roots were then quickly placed in an airtight container of 1000 ml. 150 ml of acetylene was then injected in the container, and a 6.5 ml gas sample was taken by a syringe 5, 10 and 15 minutes after incubation, and the samples were then subjected to gas chromatography. It has been established that acetylene reduction is linear for about 20 minutes from the acetylene introduction. The results obtained after 15 minutes are shown in Table 8.

Table 8
Effect of betaine application on the nitrogen fixation of soybean

betaine concentration (kg/ha)	ethylene concentration ($\mu\text{mol}/2$ plants)			
	Cv. Cook		Cv. Biloxi	
	dry stress	normal	dry stress	normal
0 (control)	0.29	0.55	0.42	0.68
3	0.60	0.68	0.53	0.79
6	0.34	0.52	0.35	0.38

The application of betaine at the rate of 3 kg/ha thus clearly increases the nitrogen fixation of plants.

Example 9**Effect of betaine application on soybean yield**

5 The acetylene reduction test described in
 Example 8 was repeated in greenhouses utilizing the
 betaine rate of 0.1M and cultivar Biloxi. The experiment
 was conducted by closing plant roots (2 per pot) in a
 glass container (1 l) and by sucking 150 ml of air out
 of the container, whereafter the air was replaced with
 a corresponding amount of acetylene gas in the manner
 10 described in Example 8. (Reference: Denison, R.F.,
 Sinclair, T.R., Zobel, R.W., Johnson, M.N. & Drake, G.M.
 1983. *A non-destructive field assay for soybean nitrogen
 fixation by acetylene reduction*. Plant & Soil 70; 173-
 182; Vessey, J.K. 1994. *Measurement of nitrogenase
 activity in legume root nodules; In defense of the
 acetylene reduction assay*. Plant & Soil 158; 151-162).

15 The nitrogen fixation of soybeans of 4 weeks
 was assayed in greenhouse tests 2 days after the betaine
 application. The results are shown in Table 9.

20

Table 9

Effect of betaine application on the nitrogen fixation
 of soybean

betaine concentration (M)	ethylene concentration (ppm)	
	normal	water stress
0 (control)	3.029	2.193
0.1	3.642	2.690

30

Example 10**Effect of betaine application on soybean yield**

The effect of betaine application on the photosynthesis of soybean was examined in greenhouses utilizing simulated water stress conditions.

5 Six (inoculated) seeds of nodulating or 15 (inoculated) seeds of non-nodulating soybean cultivar were sown in 5 l plastic pots containing a mixture of peat, vermiculite and sand in the ratio of 1:2:1. After
10 seedling emergence the plants were thinned to 3 per pot. Half of the pots used in the experiment contained the nodulating cultivar and half the non-nodulating cultivar. Water stress (pF3) was imposed on the plants 15 days after seedling emergence. The water-stressed
15 plants were divided into three groups, one of which (control) was treated with distilled water, the second one was sprayed with betaine at the rate of 2 kg/ha and the third one with betaine at the rate of 6 kg/ha a day after stress imposition. The photosynthetic activity of
20 the plants was determined with the Li-cor Li-1600-Steady State Porometer. The apparatus and its use are described in the following references: Campbell, G.S. 1975. *Steady-state diffusion porometers*. In: Measurement of stomatal aperture and diffusive resistance. Coll. Agric. Res. Center Bull. 809. p. 20. Washington State Univ. Pullman, Wash, and Bingham, G.E. & Coyne, P.I. 1977. *A portable, temperature-controlled steady-state porometer for field measurements of transpiration and photosynthesis*. *Photosynthetica* 11(1): 148-160. The
25 results are shown in Table 10.
30

Table 10

Effect of betaine application on the photosynthetic activity of soybean

betaine concentration (kg/ha)	photosynthetic activity ($\mu\text{mol m}^{-2}\text{s}^{-1}$)									
	nodulating cultivar					non-nodulating cultivar				
	normal	% of control	dry stress	% of control	normal	% of control	dry stress	% of control	dry stress	% of control
0 (control)	33	100	10	100	33	100	9	100	33	100
2	39	120	18	180	33	100	26	100	289	289
6	36	110	15	150	37	112	26	112	289	289

Betaine considerably improved the photosynthetic activity of soybean both under normal and water stress conditions with both application rates.

5 **Example 11**

Effect of betaine application on the growth of soybean

 The effect of betaine on the photosynthesis of soybean and on the water potential situation of soybean plant leaves was examined in greenhouses with the betaine concentrations of 0 (control), 0.05M, 0.10M and 0.15M. The soybean cultivar was Biloxi. At the time of spraying, the plants were about six weeks old and the measurements were performed five days after the spraying. The photosynthesis and the water potential of the plants, i.e. the stomatal resistance and stomatal conductance and the temperature difference of leaves were measured with the Li-cor Li 6200 Portable Photosynthesis System. The system is based on a method described by Ball et al. (*A Model predicting stomatal conductance and its contribution to the control of photosynthesis under different conditions*. Progress in Photosynthesis Research IV. Martinus Nijhoff, Dordrecht, The Netherlands, 1987, pp. 221-224, (Ed.) J. Biggins).
Water potential parameters indicate the state of opening or closing of the guard cells of the plant. High resistance and low conductance mean that the plant has a poor intake of carbon dioxide and is stressed. The numerical results are shown in Table 11, a graphical illustration is provided in Figure 3.

Table 11

Effect of betaine on the photosynthesis and leaf water potential of soybean

5	betaine concentration (M)	photo-synthesis ($\mu\text{molm}^2\text{s}^{-1}$)	stomatal conductance (ms^{-1})	stomatal resistance (scm^{-1})	leaf temperature difference
10	0 (control)	2.70	0.02	18.16	-2.81
	0.05	4.01	0.05	11.21	-3.01
	0.10	14.01	0.26	6.30	-1.55
	0.15	12.83	0.12	4.46	-1.72

15 The results clearly show that adding betaine can reduce the stress of plants. The photosynthetic activity of the plant thereby increases, thus leading to a higher yield.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Exogenous use of betaine to improve the yield of grain legumes.
- 5 2. Exogenous use of betaine to reduce the antinutrient content of grain legumes.
3. Use of betaine according to claim 2, wherein said legume is lupin and the antinutrient is the alkaloid content.
- 10 4. Use according to any one of claims 1 to 3, wherein the use of betaine is under stress conditions.
5. Use according to claim 4, wherein the stress conditions comprise high or low temperatures, drought or high salinity.
- 15 6. Use according to any one of claims 1 to 5, wherein the amount of betaine used is about 0.1 to 20 kg/ha.
7. Use according to claim 6, wherein the amount of betaine used is about 1 to 6 kg/ha.
- 20 8. A method for improving the yield of grain legumes comprising the step of exogenously applying betaine to growing grain legumes.
9. A method for reducing the antinutrient content of grain legumes comprising the step of exogenously applying betaine to growing grain legumes.
- 25 10. A method according to claim 9, wherein said grain legumes are lupins and the antinutrient is the alkaloid content.
11. A method according to any one of claims 8 to 30 10, wherein the betaine is applied to grain legumes growing under stress conditions.
12. A method according to any one of claims 8 to 11, wherein the stress conditions comprise high or low temperatures, drought or high salinity.
- 35 13. A method according to any one of claims 8 to 12, wherein the betaine is administered at least once during the growing season.



14. A method according to any one of claims 8 to 13, wherein the betaine is administered together with a pesticide or a fertilizer.

5 15. A method according to any one of claims 8 to 14, wherein the betaine is administered in a single dose at an early stage of growth.

16. A method according to any one of claims 8 to 15, wherein the betaine is used in an amount of about 0.1 to 20 kg/ha.

10 17. A method according to any one of claims 8 to 16, wherein the betaine is used in an amount of about 1 to 6 kg/ha.

15 18. Grain legumes and the parts thereof, especially the seed yield, obtained by a method according to any one of claims 8 to 17.

19. Grain legumes treated exogenously with betaine, and the products thereof.

20 20. An invention according to any one of claims 1 to 19, wherein the grain legume is selected from the group consisting of lupin, pea, soybean, faba bean and green bean.

21. Use according to claim 1 substantially as hereinbefore described with reference to any one of the examples.

25 22. A method according to claim 8 substantially as hereinbefore described with reference to any one of the examples.

Dated this 29 day of January 1999

30 CULTOR OY

By their Patent Attorneys

GRIFFITH HACK

Fellows Institute of Patent and

Trade Mark Attorneys of Australia



FIG. 1

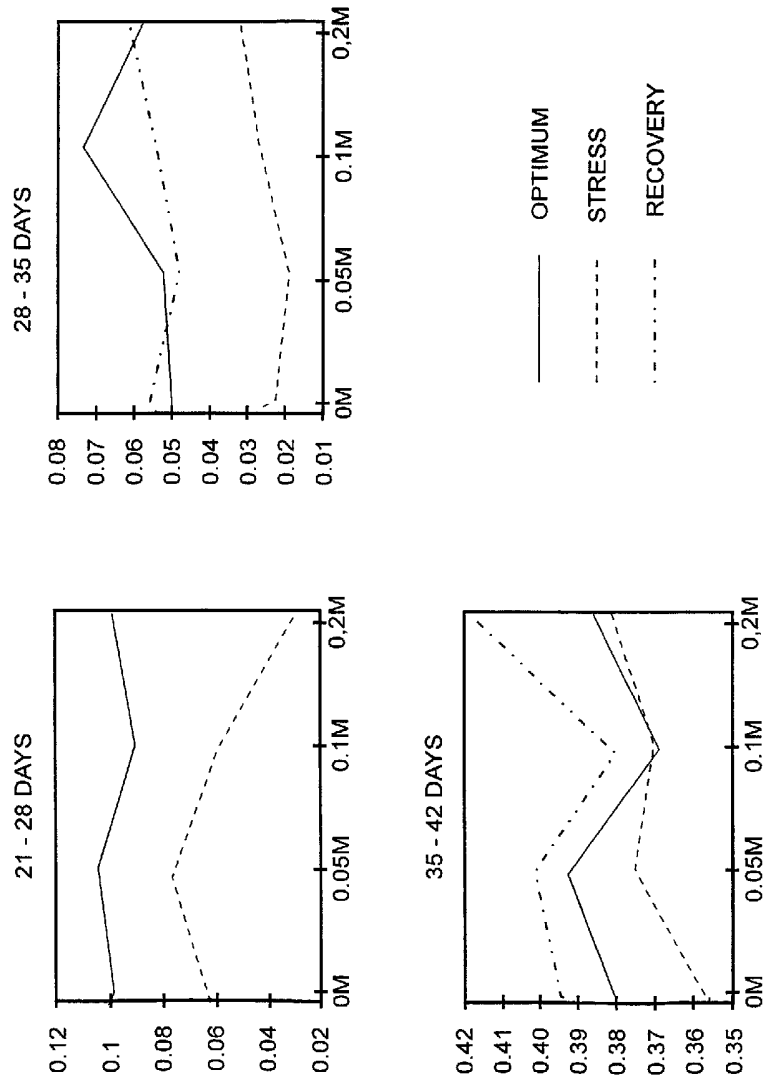


FIG. 2

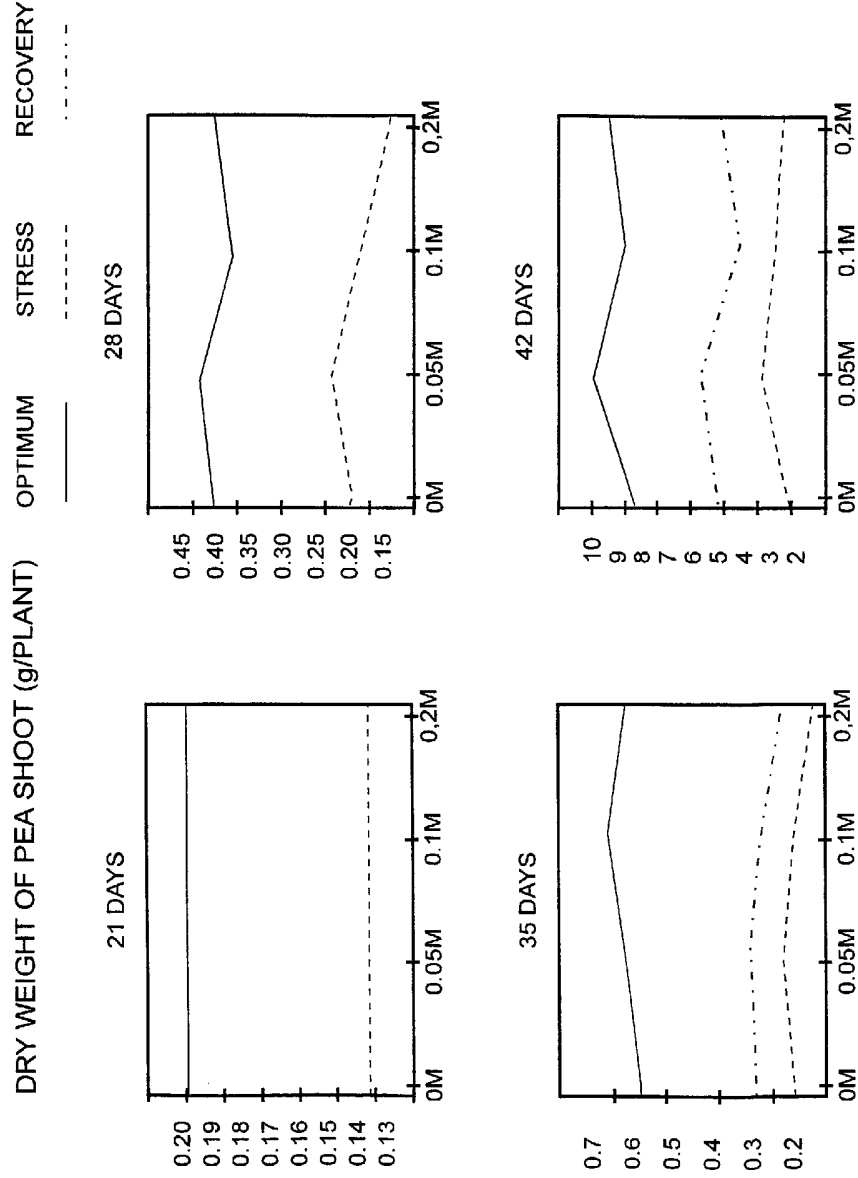


FIG. 3

