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(54) Title: METHOD FOR THE TREATMENT OF SEEDS WITH BETAINES

(57) Abstract

A method of treating seeds with betaine by either soaking the seeds in a betaine solution containing 0.34 M or lower concentration of betaine or coating the seeds with betaine at a ratio of 1-10 betaine weight per seed rate, prior to planting the seeds. Treating the seeds with betaine enhances seedling growth and protects the germinating seeds against adverse conditions. This method is commercially significant in areas where there is drought or high salinity.

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TITLE

METHOD FOR THE TREATMENT OF SEEDS WITH BETAINES

FIELD OF THE INVENTION

THIS INVENTION relates to a method for the treatment of seeds with betaines.

PRIOR ART

Under conditions of environmental stress plants accumulate a large quantity of organic solutes containing nitrogen. One group of these solutes is broadly classified as betaines. At least 12 different betaines occur in plants. A list of the major betaines is given in Table 1 of the following reference, i.e. Wyn Jones and Storey, Betaines. In The Physiology and Biochemistry of Drought Assistance on Plants. Paleg and Aspinall (eds.) Academic Press. Sydney 1981, pp171-204.

Glycinebetaine is the most common of the betaines and occurs widely in plants. A number of plants from a variety of families have been identified which have accumulated proline (Aspinall and Paleg. Proline accumulation: physiological aspects. In The Physiology and Biochemistry of Drought Resistance in Plants. Paleg and Aspinall (eds.) Academic Press. Sydney 1981, pp97-130) and glycinebetaine (see Wyn Jones and Storey reference above). As well, betaines accumulate in native Australian plants such as *Melaleuca spp.* (Poljakoff-Mayber *et al.*, 1987. Aust. J. Plant Physiol. **14** 341-50, Jones *et al.*, 1987. Phytochemistry **26** 3343-3344 and Naidu *et al.*, 1987. Aust. J. Plant Physiol. **14** 669-677).

In the laboratory betaines have been shown to protect plant processes against cold, heat, drought and salinity. Betaines have been shown to protect enzymes against denaturation through stabilization of the native enzyme structure resulting from molecular crowding (Winzor *et al.*, 1992. Archives Biochemistry Biophysics **296** 102-107). Betaines are also involved with other cellular components such as with protecting membrane proteins against dehydration and conformational changes

(Jolivet *et al.*, 1983. Z. Pflanzenphysiol **109** 171-180).

Because betaines have been shown to provide some protection to plants in adverse environments, they have been used to treat soils and growth media, as well as being sprayed onto the leaves of plants. German Patent Specification No. 2808365 discloses an organic mineral soil improver which includes betaine. Both British Patent Specification No. 2180529 and U.S. Patent Specification No. 4818268 describe osmoprotectant particles for enhancing the growth of mushrooms. The osmoprotectant particles comprise a carrier particle having recessed attachment sites and osmoprotectant droplets having a core of a phospholipid material surrounded by a layer of an osmoprotectant material which includes at least one betaine. The droplets are attached to the recessed attachment sites of the carrier particle. The osmoprotectant particles may be added either to the compost for the mushrooms or be brought into direct contact with immature spawn prior to planting.

Japanese Patent Specification No. 01208386 discloses a fertiliser containing betaine that can be applied to paddy fields for hydroponic cultivation or may be sprayed over leaves of plants. As well, the fertiliser can be in the form of grains or pellets.

In Bodapati *et al.* (Bodapati *et al.*, 1992. 32nd Annual General Meeting of the Australian Society of Plant Physiologists Abstract No. 39), the researchers reported an increase in grain yield of cold-stressed buckwheat in field conditions with the foliar application of glycinebetaine. Initial experiments involved the growing of plants in glass house conditions for three weeks at 20°C. Glycinebetaine or proline at 0, 5, 15 or 50 mM was applied twice to the foliage. Three days after application the plants were exposed to 1°C for two days. Glycinebetaine treated plants maintained higher levels of both relative water content and chlorophyll-b than control plants. Proline application provided some positive influence on chlorophyll-b content only. For the field trials 50 mM glycinebetaine was applied to the foliage resulting in a 15% increase in

grain yield.

As well as treatment of soil and foliage, seeds have been treated with a betaine prior to germination. A group of researchers treated carrot and celery seed at -1.0 MPa (approximately 0.4 M) and onion and leek seed at -1.5 MPa (approximately 0.6 M) with betaines and compared with a control treatment with seed germinating in water for 14 days at 15°C. They found the percentage of glycinebetaine treated leeks, celery and onion seeds germinating was not affected by the treatment. However, the percentage of glycinebetaine treated carrot seeds germinating was reduced. As well, germination time for all four species was reduced compared with controls (Gray *et al.*, 1991. Seed Sci & Technol. **19** 581-590).

Another group working with *Kosteletzkya virginica* found that the application of glycinebetaine or proline did not improve germination under saline conditions. Glycinebetaine or proline was added to the germination medium at 10 mM concentration and the seeds were incubated in 12 hour alternating temperatures of 28/18°C in the dark. After four days incubation, the germination of *Kosteletzkya virginica* seeds in a non-saline germinating medium showed no significant effect with the addition of proline or glycinebetaine. However, in the presence of 100 mM NaCl in the germinating medium, the germination of seeds in medium also containing 10 mM glycinebetaine or proline were inhibited compared with the germination of seeds in medium without glycinebetaine or proline (Poljakoff-Mayber *et al.*, 1994. American Journal of Botany **81** 54-59).

SUMMARY OF THE INVENTION

The present invention results from the surprising discovery that the application of a betaine at a defined concentration to seeds enhances seedling growth and protects the seeds against the effects of adverse conditions in a stressful environment during germination. This was hitherto unknown before and indeed the prior art teaches that the application of a betaine to a seed had no effect on seedling growth during

germination and may even be inhibitory.

Thus it is an object of the present invention to provide a method of treating the seeds so to enhance seedling growth during germination and or protect germinating seeds against adverse conditions.

In one aspect, the invention provides a method for treating a seed to enhance seedling growth and/or protect against environmental stress during germination by treating the seed with betaine prior to planting whereby the seed is immersed in a solution of the betaine and said solution has a 0.34 M or lower betaine concentration, or the seed is coated with a solid form of the betaine at a ratio of 1-10 betaine weight per seed weight.

The seed may be obtained from any seed producing plant including those from the groups *gymnospermae* and *angiospermae*. The seed may be from a monocotyledon plant such as wheat or a dicotyledon plant such as vegetable legumes, fruit trees, and ornamental plants (e.g. flowers). The seed may be obtained from fodder legumes such as *Desmanthus virgatus* and *Leucaena leucocephala*, or *Trifolium repens*.

The seed may have a hard seed coat or a soft seed coat. Many varieties of dicotyledon plants have seeds with hard seed coats, whereas only some monocotyledon plants have seeds with hard seed coats. Suitable seeds with a hard seed coat include lupins, lecurne, castor, *Leucaena leucocephala* and *Desmanthus virgatus* seeds. Suitable seeds with a soft seed coat include peas, raddish, cauliflower and some beans. Soft coated seeds are usually permeable except when they are in a dormant state. Soft coated seeds in a dormant state are prevented from germinating by the accumulation of germinating inhibitors and require long soaking times in which to allow the inhibitors to leach from the seed. Seeds including those with hard and soft seed coats, which are not permeable to preparations of the betaine or betaine analogue may be made substantially permeable by roughening or scarifying the surface of the seed coat prior to treating the seed with the betaine. Scarification may be achieved by mechanical means, using hot

or boiling water, or subjecting the seed to acid such as sulphuric acid or alkali treatment. Scarification is preferably achieved by mechanical means. A mechanical scarifier may be fitted with coarse sand paper or the like. The seed is preferably scarified until the seed coat is no longer glossy and scars appear on the seed coat. Not all seeds require scarification and of the seeds used as examples below, namely wheat, cotton, tomato and *Desmanthus*, only *Desmanthus* may require scarification.

Environmental stress includes water stress (flood and drought), excess NaCl (salinity), temperature extremes (heat and cold), pH extremes (acidic and alkaline soils) and heavy metal toxicity.

The term "germination" includes the development into a plant or individual from a seed.

Betaine includes amino acids where the nitrogen is fully or partially methylated. Some betaines may have sulphur substituted for nitrogen. A betaine may be any molecule with the general formula:

$$(CH_3)_n - N^+ - (CH_2)_m - COO^-$$

where n may be 1, but preferably 2 (for cyclic betaines) and 3 (for straight chain compounds) and m is at least 1. Betaine preferably includes glycinebetaine and those betaines listed in Table 1.

The betaine concentration in solution may depend on the type of seed, whether the seed is or has been made permeable, the betaine type, and the length of time the seed is immersed in the solution. The duration of soaking is preferably for at least two hours but may not be greater than 12 hrs. Soaking the seed in the betaine solution may facilitate the entry of the betaine into the seed whereupon it may be available for use by the germinating seed. In the case of wheat, it has been demonstrated that 40-80 g betaine per kg of seed in 2 litres of water and immersed for 12 hrs prior to drying and planting in pots produced a germination rate that was more than double the germination rate of the control under 0.2 M salinity conditions. As well, there was a four fold

increase in wheat grain yield compared with control.

The solution of betaine may also contain wetting agents and/or surfactants which assist in permeating the seed.

Soaking may occur at any temperature but preferably at 25°C.

The concentration of 0.34 M approximately corresponds to 40 g/L of glycinebetaine which is the highest concentration at which beneficial effects were observed. Beneficial effects were also noted with the use of lower concentrations. It is suggested that high concentrations of betaine are viscous and have lower osmotic potential and inhibit the uptake of betaine by the seed.

After treating the seed with the preparation of betaine, the seed may be dried if it is not to be used immediately. The seed is preferably dried to a stage where it can be stored in normal commercial storage conditions without loosing its ability to benefit from the treatment upon germination. The seed is preferably dried to a stage whereupon storing it does not rot. The seed may be dried to a stage where it contains only about 10% moisture.

After immersion or soaking the seed may be dried and may be coated with a drying agent. This may be necessary as betaine is hydroscopic. Suitable drying agents include lime, gypsum, dolomite, rock phosphate and several clay minerals (montmorillonite and vermiculite). The betaine soaked seed may also be coated with an adhesive to ensure the retention of betaine. Suitable adhesives include methyl cellulose and gum arbica which comprises a mixture of plant extracted gums. A further coating of a drying agent may be added. These coatings may be added stepwise or a drying agent and adhesive may be added together. Other coatings may be added to facilitate the slow release of betaine. A suitable example may be a betaine-soaked seed coated in lime and methyl cellulose.

A solid form of betaine may consist of betaine in a powder. Other additives such as fillers, adhesives and drying agents may also be

added. Preferably a seed is coated with an adhesive, then a betaine coat and then coats of a drying agent and an adhesive. Betaine may be mixed with inert fillers such as fine sand. Other additives may be included to effect slow release of the betaine. Fertiliser may be combined with betaine coated seed. With the onset of rain or irrigation, the betaine coating the seed will become available to be absorbed by the seed or roots. In the examples below, betaine is applied to the soil simulating release from the coat material. With respect to these seeds, it was estimated the amount of betaine required was approximately 1, 1, 5 and 3-5 kg per kg of seed for wheat, cotton, *Desmanthus virgatus* and tomato respectively.

The methods of applying the various coatings are standard techniques (Scott, 1989, Advances in Agronomy, Ed. Brady, Vol. 42, pp 44-77, Academic Press, San Diego, U.S.A.).

Irrespective of whether the betaine is applied in a solution or coating on the seed, the concentration of betaine available for use by the seed is important and is achieved by immersing the seed in betaine solution of 0.34 M or lower concentration or by coating the seed with betaine at an amount of 1-10 betaine weight/seed weight.

In a second aspect, the invention provides a seed treated by the aforementioned method.

Reference may now be made to various preferred embodiments and it should be noted that the references to specific seeds, betaines and concentrations are given by way of example only.

EXPERIMENTAL

EFFECT OF BETAINE SEED TREATMENT ON GERMINATION, GROWTH AND YIELD OF WHEAT, COTTON, TOMATO AND *DESMANTHUS* GROWN IN SALINE OR WATER STRESSED CONDITIONS

1.0 WHEAT

1.1 *Seed soaking time*

The viability of soaked and re-dried seed depends on the length of soaking. An experiment was conducted to find an optimum time required to soak seed in water without any adverse effects on germination and vigour.

Ten gram lots of wheat (*Triticum aestivum* cv. Hartog) seed was soaked in small beakers with 20 ml of de-ionised water at 20°C for 0, 2, 4, 8, and 12 hrs with four replications. At the end of each soaking period, seed was weighed and re-dried for 4 hrs at 45°C. Germination and vigour tests were done in petri dishes with 11 cm diameter. Ten seeds were placed in each petri dish on a filter paper with 12 ml of de-ionised water. The experiment was conducted in an incubator with 22/18°C day/night temperature with 12 hrs of photo period with a photon flux density of 75 μEm^{-2}. At the end of 3 days of germination, shoot and root lengths were measured.

Wheat germination and vigour which is measured as shoot and root lengths showed no difference in relation to even 12 hrs of soaking (Table 2). It was also clear that imbibition was complete by 8 hrs soaking. These results suggests that soaking of seed with a betaine solution will have no adverse effect up to 12 hrs of soaking and re-drying.

1.2 *Effect of betaine on germination of salinised wheat in petri dishes*

Seed was soaked in 5, 10, 20, 30 or 40 g/L solution of betaine for 12 hrs. The weight of the betaine solution added was twice the weight of the wheat seed. Then the seed was rapidly dried in a fan forced oven at 45°C for 4 hrs. This seed with untreated control seed was used in petri dish salinity experiment. The conditions for this experiment were same as in 1.1 except that the germinating medium, contained 3 levels of salt solutions, 0, 0.15, and 0.2 M. Seedling height and root lengths were measured 10 days after the start of the imbibition.

Betaine showed no significant effect on the shoot or root

length of wheat when there was no salt in the germination medium. However, at both 0.15 and 0.20 M salinity levels, seedling vigour was significantly reduced in the absence of betaine seed treatment. On the other hand, seed imbibition of betaine increased shoot length at both the salinities tested. Betaine effect was maximum at 20 g/L and this effect starts reducing with higher betaine levels.

1.3 ***Effect of betaine imbibition on the germination of wheat in salinised pots***

Plastic pots (6") fitted with plastic lining bags to cover the drainage holes were filled with 1.8 kg Samford loam. Each pot was watered with 450 ml of 0, 0.15 or 0.2 M NaCl solution with 4 replications. Betaine levels of 20, 40, and 60 g/L were imbibed in a similar manner to the one described in section 1.2. Ten seeds were planted per pot and maintained in a growth cabinet with 22/18°Cday/night, 400 μEm^{-2}. Germination and emergence counts were taken every day for the first 10 days. Then plants were fertilised with Aquasol at 8 g/L as per the manufacturers recommendation. The pots were weighed every alternative day and water used up is added to make up 450 ml/pot. Plants were maintained until the maturity under these conditions. The results are shown in FIGS. 1-3.

Wheat showed a germination of 98% in non-saline soil (FIG. 2). This was reduced significantly by 0.15 and 0.2 M salinity to 52 and 25%. Betaine imbibition showed no difference on germination when there was no salinity. All betaine levels significantly increased germination at both at 0.15 and 0.2 M salt levels (FIG.2). Maximum benefit was achieved with betaine at 40 g/L and 0.15 M salinity. Further increase in betaine level showed a reducing trend in germination. However, at 0.2 M salinity, betaine was showing an increasing trend even at the highest betaine concentration of 60 g/L. This indicates that a specific soil salinity requires a specific dose range of betaine for useful effect on germination.

The data from the same experiment shows that salinity significantly reduces grain yield (FIG. 3). At 0.15 M salinity grain yield

was significantly increased by about 30% with the use of betaine compared to the use of non-betaine imbibed seed. This effect was very dramatic at 0.2 M salinity level where, the yield increase was about 400% more than with the use of non-betaine imbibed seed. At both the salinities the use of any higher levels of betaine was not beneficial.

1.4 ***Effect of betaine on wheat in saline medium in petri dishes***

This experiment is similar to the one described in the section 1.2 except that betaine at 0, 1, 2, 4, 8, 16 and 20 g/L was added in all combinations to the solutions containing 0, 0.1, 0.15 and 0.20 M NaCl with 4 replications. Seedling height and root lengths were measured 10 days after the start of the experiment.

Presence of betaine in germination medium showed no response on seedling growth of non-salinised or salinised seedlings with 0.1 M salt. However, at 0.15 and 0.2 M salt levels, betaine at 1 or 2 g/L resulted in a very significant increase in shoot length compared to non-betaine treated and salinised treatments (FIG. 4). Beneficial effect reaches a peak at 2 g/L and further levels actually reduce the shoot growth at both salinities.

1.5 ***Wheat germination in saline soil when betaine is added to the soil***

The experimental protocol and conditions of plant growth are similar to the one described in the section 1.3 except that in each pot, 10 unimbibed and untreated seed were planted with betaine at 0, 1, 2, 4, and 8 g/L in all combinations with 0, 0.1 and 0.15 M salinity. Seedling emergence was noted every day for 10 days.

The results of the previous petri dish experiments were tested in pot situation. In non-saline soil the percentage of germination was 87.5% and this was drastically reduced to 42% and 35% in response to salinity of 0.1 and 0.15 M respectively. Addition of 1 or 2 g/L of betaine significantly increased germination to almost non-saline conditions at 0.15

M salinity (FIG. 5). However, any higher levels of betaine seem to have reduced germination at 0.2 M salinity.

1.6 *Effect of betaine on water stressed wheat seedlings in petri dishes*

Betaine solution of 0, 1, 2, 4, 8, 16, and 20 g/L was mixed in 20% PEG (MW 4,000) solution. Each petri dish with 10 wheat seed was treated with one of the concentrations of betaine in 12 ml of 20% PEG solution and germination test was performed and shoot and root lengths were measured 10 days after the initiation of the experiment.

Presence of 1 or 2 g/L of betaine in PEG medium significantly increased growth of water (PEG) stressed seedlings. Any higher levels of betaine actually reduced the growth of seedlings significantly (FIG. 6)

1.7 *Effect of various betaines on the growth of wheat seedlings*

Glycinebetaine (B), N-methyl proline (MP), N-dimethyl proline or stachydrine(S), N-methyl-*trans*-4-hydroxy-proline (MHP), N-dimethyl-*trans*-4-hydroxy-proline (DHP), Trigonelline (T) were the six betaine/betaine analogues tested in this experiment. The compounds were applied at 10, 20 and 40 mM. 12 ml of this solution was placed in petri dishes with 10 wheat seed as described in the section 1.2. Shoot and root lengths were measured after ten days. Only one salinity concentration of 0.2M was used.

Six different betaines/betaine analogues, including glycinebetaine, were tested on the shoot and root growth of 0.2 M salinised wheat seedlings in petri dishes. Only N-methyl proline at 10 mM showed about 11% increase in root length. Trigonelline showed reduced root length by about 13%. N-methyl proline showed no response on shoot length. At this salinity, the other four betaine analogues increased shoot length by as much as 68% compared to control, however, the concentration required by each compound to obtain this effect varied from

10-40 mM (Table 3).

2. COTTON

2.1 *Effect of betaine on the germination and growth of salinised cotton in pots*

Plastic pots (6") fitted with plastic lining bags to cover the drainage holes were filled with 1.8 kg Samford loam. Betaine at 0, 1, 2 and 4 g/L was mixed in all combinations with salinity levels of 0, 0.1, 0.15 and 0.2 M. 400 ml of the solution with each of the combinations of betaine and salt was applied to each pot. Ten unimbibed and untreated cotton (*Gossypium hirsutum* cv. Siokra) seed were planted in each pot. The experiment was conducted in naturally lit glasshouse (Conpol) at Samford with temperature control (max 35°C & min 20°C). Germination counts were taken for 10 consecutive days. Later, pots were thinned to 2 plants /pot where possible and fertilised with 100 ml of Aquasol solution (8 g/L). Pots were weighed on alternative days and the lost water was added to 400 ml to maintain the salinity level relatively constant.

Two months after planting, all the leaves in each pot were harvested to measure leaf area using automatic leaf area meter. Stem, and root lengths and the oven dry weights of leaf and shoot (stem + leaf wt) were measured.

Non-salinised cotton showed a percentage of germination of above 85%. Salinity had a drastic effect on germination, reducing it to 77%, 37% and 2.5% in response to 0.1, 0.15 and 0.2 M NaCl respectively. At all levels of salinity, betaine increased germination (FIG. 7) compared with non-betaine treated controls. At 0.1 M salinity, betaine application at 1 g/L restored germination back to the non-salinised control level. At 0.15 M and 0.2 M NaCl, 2 g/L of betaine increased germination more than non-betaine treated salinised treatments. At these salinities, any further increase in betaine level showed the tendency of reducing germination from its peak value at the lower betaine levels.

Leaf area of cotton (FIG. 8) was significantly increased by

about 20% in non-salinised controls and at 0.1 M salinity, in response to 1 g/L betaine application. At the highest salinity (0.2 M NaCl) the leaf area was increased by more than 3 and 6 fold in response to 1 and 2 g/L betaine application, respectively. Leaf dry wt (FIG. 9) also followed similar trends in response to betaine application in saline soil. Shoot dry wt (FIG. 10) was increased in control as well as in 0.15 M salinity by about 40%. At the highest salinity (0.2 M NaCl) dry matter was increased by about 5 times in response to 2 g/L of betaine. Shoot and root lengths were not influenced by betaine application.

2.2 ***Effect of betaine on water stressed cotton***.

Plastic pots (8") fitted with plastic liner bags were filled with 4.5 kg of Samford loam. 900 ml of solution containing 0, 2, 4, 8 and 12 g/L of betaine was applied to the pots. Ten cotton (cv. Siokra) seed were planted and then the seedlings were thinned down to 5 after germination. When seedlings were about 4 weeks, 200 ml of Vermiculite was added to the soil surface in each pot. Final watering was done to make sure that each pot contained 900 ml of water and then further watering was stopped. Four weeks after this, relative water content of the youngest and most expanded leaf was measured.

When water was withheld from cotton seedlings, control plants reached to about 40% relative water content (RWC) which is very close to the death point (32%) for some crop plants. On the other hand, betaine application from 2 to 12 g/L increased the RWC in a linear fashion to about 75% (FIG. 11) which is similar to the value that plants under moderate stress will contain. This suggests that betaine increases drought tolerance or postpones the occurrence of severe water stress symptoms.

2.3 ***Effect of betaine on water use and growth of cotton***

Plant culture for this experiment is exactly same as the one described in the section 2.2. Pots were weighed once in 2 or 3 days to measure water use. In this experiment there were two levels of watering:

(A) when water content reaches 0.1 kg/pot, well watered plants received watering to field capacity (water content of 0.9 kg/pot); and (B) plants receiving limited watering were maintained only to 0.4 kg of water/pot when the water content fell to 0.1 kg/pot. Care was taken that vermiculite was added to the pot soil surface to cut down evaporation. Blank pots without plants were also maintained to estimate the unavoidable evaporation from the vermiculite surface. Evaporative loss has been deducted from the gross water use of plants to arrive at the net water used in transpiration.

When the plants were 2 months old, shoot length and weight, root length and weight, and leaf area of the plants were measured.

When plants were well watered (i.e. watered to field capacity when reached wilting point), betaine application at 4 g/L actually resulted in more water consumption than controls for 4 days (FIG. 12) and with the limitation of water availability, water use has started declining than non-betaine treated controls. Betaine application at 8 and 12 g/L resulted in significantly lower water use throughout the period and reaching about 48% less water use compared with the control. Similar pattern of water use was also noted in limited water application treatment also (FIG. 13).

Betaine application reduced shoot length in both well- and limited-watering treatments (Table 4) on a concentration dependent manner. Although shoot weight was also reduced in a similar pattern to the shoot length, the shoot length reduction was to a lesser magnitude. (Table 4). Root length was not affected by betaine application (data not included), however, root weight of both well- and limited-watered plants was reduced on a concentration dependent manner (Table 5). This suggests that root diameter has reduced in response to betaine application. Leaf area also reduced in both the watering treatments in response to betaine application. (FIG. 14).

3. DESMANTHUS

3.1 *Effect of betaine on Desmanthus virgatus in petri dishes*

Desmanthus virgatus seed was scarified in a mechanical scarifier fitted with a coarse sand paper. The process was repeated until the seed was no longer glossy and had scars on the seed coat. Scarified seed was treated with glycinebetaine whereby one gram of scarified seed was soaked in 10 ml of one of several glycinebetaine solutions. Glycinebetaine solutions containing 0, 2, 4, 8 and 12 gram glycinebetaine per litre of distilled water (corresponding to 0, 17 x 10^{-2} M, 3.4 x 10^{-2} M, 6.8 x 10^{-2} M and 1.0 x 10 M respectively) were used. The seeds were soaked for 12 hours at 25°C then immediately dried in a dehydrator at 45°C for about 3 hours.

Ten treated seeds were placed in a petri dish containing a base of filter paper. The treated seeds were watered with 13 ml of distilled water or 0.15 M NaCl solution. The treated seeds were incubated in an incubator at 30°C for 4 days in the dark before the length of the root (radicle) and shoot (plumule) were measured. The germination count of the seeds was above 90% when distilled water was used as the germinating medium in the petri dishes. The growth of seedlings as indicated by the length of the shoot and root is shown in FIG 15.

Radicle length increased by more than three-fold with seeds treated with 12 g/L glycinebetaine compared with non-treated seeds when watered with either distilled water or 0.15 M NaCl solution. The beneficial effects of betaine was shown to be not dependant on salinity alone.

The shoot length of 12 g/L glycinebetaine treated seeds was approximately 1.6 times the shoot length of non-treated seeds when watered with either distilled water or 0.15M NaCl. However, the shoot length of 12 g/L glycinebetaine treated seeds watered with distilled water was approximately 1.3 times the shoot length of similarly treated seeds but watered with 0.15 M NaCl. It should be noted though that the shoot length of 12 g/L glycinebetaine treated seeds watered with 0.15 M saline was longer than the shoot length of non-treated seeds watered with

distilled water.

One conclusion from the experiment was that seeds treated with 12 g/L glycinebetaine by soaking for 12 hrs at 25°C and watered with 0.15 M NaCl can germinate in what would be normally growth restrictive conditions.

3.2 ***Effect of betaine with soaking of Desmantus virgatus seeds for various times***

Desmanthus virgatus seed was scarified in a mechanical scarifier fitted with a coarse sand paper. The process was repeated until the seed was no longer glossy and had scars on the seed coat. Scarified seed was treated with glycinebetaine whereby one gram of scarified seed was soaked in 10 ml of one of several glycinebetaine solutions. Glycinebetaine solutions containing 0, 12, 24 and 36 gram glycinebetaine per litre of distilled water (corresponding to 0, 1.0 x 10^{-1} M, 2.0 x 10^{-1} M and 3.0 x 10^{-1} M respectively) were used. The seeds were soaked for 2 or 4 hrs at 25°C then immediately dried in a dehydrator at 45°C for about 3 hrs.

Ten treated seeds were placed in a petri dish containing a base of filter paper. The treated seeds were watered with 13 ml of distilled water or 0.15 M NaCl solution. The treated seeds were incubated at 30°C for 4 days in the dark before the lengths of the root (radicle) and shoot (plumule) were measured. The germination counts of the seed was above 90% when distilled water were used as the germinating medium in the petri dishes. The results of the experiment are shown in Tables 6 and 7.

Seeds that were soaked in the various betaine solutions for 2 hrs did not show significant differences in root length, shoot length and seedling weight during germination compared with controls. This was observed when the seeds were watered with distilled water or 0.15 M NaCl.

Seeds that were soaked for 4 hrs in betaine solutions of 12 g/L and 24 g/L betaine produced shoots that were longer and seedling

weights that were greater than the corresponding controls when the seeds were watered with 0.15 M NaCl. The germination of seeds soaked for 4 hours in 36 g/L betaine solution appeared to be inhibited.

3.3 ***Effect of betaine on germination of salinised Desmanthus virgauts in pots***

Six inch plastic pots fitted with plastic bags and filled with 1.8 kg Samford loam were applied with 450 ml of solutions containing betaine at 0, 1, 2 and 4 g/L in all combinations with 0, 0.05, 0.1 and 0.2 M salt. 20 unimbibed, untreated and scarified seed were planted and water content maintained at 450 ml/pot by weighing them on alternative days for two weeks. Germination and emergence were monitored during this period.

The effect of betaine was significant only at the salinity concentration of 0.1 M (FIG. 16). Germination increased from 17% in non-betaine treated salinised treatment to 31% in response to betaine application at 1 g/L. Higher betaine application rates actually reduced germination from its peak at 1 g/L betaine application.

4. TOMATO

4.1 ***Effect of betaine on the growth of tomato seedlings***

Six inch plastic pots fitted with plastic bags and filled with 1.8 kg Samford loam were applied with 350 ml of solutions containing betaine at 0, 1, 2 and 4 g/L in all combinations with 0, 0.025, 0.05 and 0.075 M salt. 20 unimbibed and untreated tomato (*Lycopersicon esculantum* cv. Grosse Lisse) seed were planted and water content maintained at 350 ml/pot by weighing them on alternative days for two weeks. Germination and emergence were monitored during this period. Then the seedlings were thinned to 5 plants per pot and shoot dry wt was obtained 2 months after planting.

Betaine showed no significant increase in germination of tomato seed subject to 0, 0.025, 0.05 and 0.075 M salinity. However, the seedling dry weight was increased at all levels of salinity at betaine

application rates of 1 and 2 g/L and further higher application of betaine showed the reducing tendency of shoot dry weight (FIG. 17).

TABLE 1

NAMES	OTHER NAMES
Glycinebetaine	Oxyneurin, betaine
β-alaninebetaine	Homobetaine
2-trimethylamino-6-ketoheptanoate	
Prolinebetaine	Stachydrine
Proline	
N-methyl-L-proline	
Trans-4-hydroxy-N-methyl-L-proline	
Cis-3-hydroxy-N-methyl-L-proline	
(-)4-hydroxyprolinebetaine	Betonicine
(+)4-hydroxyprolinebetaine	Turicine
3-hydroxyprolinebetaine	3-oxystachydrine
Histidinebetaine	Herzynine, Ercinine
Tryptophanbetaine	Hypaphorine
2-mercaptohistidine-betaine	Ergothioneine
Pipecolatebetaine	Homostachydrine
Nicotinic acid betaine	Trigonelline

TABLE 2

Time of Soaking (h)	Root length (cm)	Shoot length (cm)	Water uptake (g/g seed)
0	6.9	3.2	0
2	6.6	3.2	2
4	6.3	3.2	6
8	6.6	3.2	9
12	6.4	3.1	9

TABLE 3

Treatment	10 mM		20 mM		40 mM	
	Root	Shoot	Root	Shoot	Root	Shoot
Control	1.5	1.8	1.5	1.8	1.5	1.8
Glycinebetaine	1.5	2.5	1.5	2.3	1.5	3.0
N-methyl proline	1.7	1.8	1.5	1.7	1.6	1.8
N-dimethyl proline or stachydrine	1.5	1.6	1.4	1.6	1.5	3.1
N-methyl-*trans*-4-hydroxy-proline	1.4	2.7	1.5	2.7	1.4	2.9
N-dimethyl-*trans*-4-hydroxy-proline	1.5	2.3	1.5	3.0	1.5	2.2
Trigonelline	1.3	1.6	1.3	2.8	1.3	1.9

TABLE 4

	Shoot length (cm)		Shoot wt (g/5 plants)	
Betaine (g/L)	Well-watered	Limited watering	Well-watered	Limited watering
0	21.2	16.5	1.88	2.14
4	20.2	15.7	1.64	1.93
8	18.8	14.7	1.47	1.72
12	16.7	14.02	1.58	1.59

TABLE 5

Betaine (g/L)	Well-watered	Limited watering
0	1.1	0.9
4	1.0	0.9
8	0.8	0.8
12	0.5	0.8

TABLE 6

TWO (2) HOURS SOAKING					
Parameter	**NaCl concentration**	**Glycinebetaine level (g/L)**			
		0	**12**	**24**	**36**
Root length (cm)	0.0 M	2.6	2.8	2.6	2.0
	0.15 M	2.1	1.7	1.5	1.3
Shoot length (cm)	0.0 M	3.8	3.9	3.8	3.3
	0.15 M	1.5	1.5	1.4	1.3
Seedling weight (g/10)	0.0 M	0.23	0.27	0.23	0.24
	0.15 M	0.20	0.20	0.21	0.21

TABLE 7

FOUR (4) HOURS SOAKING					
Parameter	**NaCl concentration**	**Glycinebetaine level (g/L)**			
		0	**12**	**24**	**36**
Root length (cm)	0.0 M	2.7	2.4	2.6	2.5
	0.15 M	1.8	1.5	1.6	1.4
Shoot length (cm)	0.0 M	3.8	3.5	3.9	3.7
	0.15 M	0.9	1.6	1.7	1.2
Seedling weight (g/10)	0.0 M	0.26	0.24	0.29	0.30
	0.15 M	0.16	0.23	0.19	0.11

LEGENDS

TABLE 1

List of major betaines.

TABLE 2

Effect of soaking time on wheat seedling root and shoot lengths and water uptake imbibition.

TABLE 3

Effect of different concentrations of betaine/betaine analogues on root and shoot growth of wheat seedlings at 0.2 M salinity.

TABLE 4

Effect of betaine and watering regimes on shoot length (cm) and shoot weight (g/5 plants).

TABLE 5

Effect of betaine and watering regimes on root weight (g/5 plants).

TABLE 6

Germination after 4 days at 30°C of *Desmanthus virgatus* seeds previously soaked in a glycinebetaine solution for two hours

TABLE 7

Germination after 4 days at 30°C of *Desmanthus virgatus* seeds previously soaked in a glycinebetaine solution for four hours

FIG. 1

Betaine imbibition on shoot growth of wheat in petri dishes

FIG. 2

Effect of betaine imbibition on wheat germination in saline soil

FIG. 3

Grain yield of salinised wheat in response to seed soaking in betaine

FIG. 4

Effect of betaine on shoot growth in saline medium of wheat in petri dishes

FIG. 5

Effect of betaine on germination of wheat in saline soil

FIG. 6

Effect of betaine of PEG on shoot growth of wheat

FIG. 7

Betaine in saline soil and cotton germination

FIG. 8

Betaine in saline soil and cotton leaf area

FIG. 9

Betaine in saline soil and cotton leaf dry wt

FIG. 10

Betaine in saline soil and cotton shoot dry wt

FIG. 11

Drought survival or relative water content (RWC) of cotton

FIG. 12

Betaine reduces water use of well-watered cotton seedlings

FIG. 13

Betaine reduces the water use of cotton plants with limited watering

FIG. 14

Reduction of leaf area of well- and limited- watered cotton plants in response to betaine application

FIG. 15

Effect of betaine seed soaking and growth of *Desmanthus virgatus* in 0.15 M NaCl solution

FIG. 16

Betaine and salinity influencing germination of *Desmanthus* in soil

FIG. 17

Dry matter accumulation of tomato seedlings in response to salinity and betaine application

CLAIMS

1. A method for treating a seed to enhance seedling growth and/or protect against environmental stress during germination by treating the seed with betaine prior to planting whereby the seed is immersed in a solution of the betaine and said solution has a 0.34 M or lower betaine concentration, or the seed is coated with a solid form of the betaine at a ratio of 1-10 betaine weight per seed weight.

2. A method as claimed in Claim 1 wherein the seed is a cereal seed, cotton seed or a seed of any other commercially significant crop.

3. A method as claimed in Claim 1 wherein the method includes one or more coatings of an adhesive and/or a drying agent and/or the betaine is glycinebetaine.

4. A seed treated with betaine whereby the seed is immersed in a solution of the betaine and said solution has a 0.34 M or lower betaine concentration, or the seed is coated with a solid form of the betaine at a ratio of 1-10 betaine weight per seed weight.

5. A seed treated by betaine whereby the seed is coated with the betaine at a ratio of 1-10 betaine weight per seed weight and one or more coatings of an adhesive and/or a drying agent.

6. A seed as claimed in Claim 6 wherein the adhesive is methyl cellulose or gum arabica and the drying agent is lime.

7. A seed treated by betaine whereby the seed is immersed in a solution containg 0.34 M or lower betaine concentration and one or more coatings of an adhesive and/or a drying agent.

8. A seed as claimed in Claims 4, 5, 6 or 7 wherein the betaine is glycinebetaine.

9. A seed as claimed in Claims 4, 5, 6, 7 or 8 wherein the seed is a seed from a commercially significant crop.

10. A wheat seed treated with betaine wherein the seed is immersed in a solution containing 0.1-0.34 M glycinebetaine.

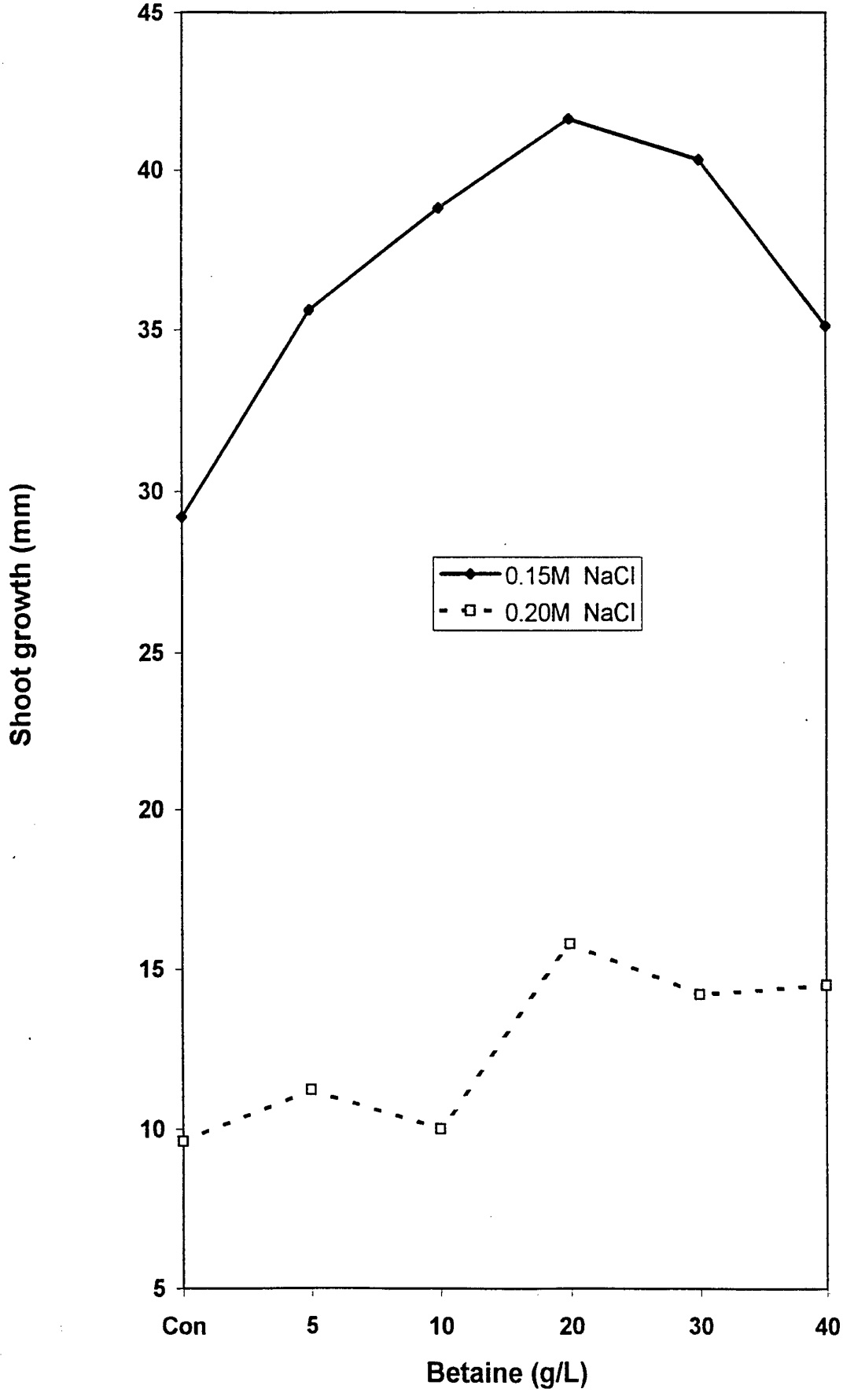
45
40
35
30
25
20
15
10
5
Shoot growth (mm)
0.15M NaCl
0.20M NaCl
Con
5
10
20
30
40
Betaine (g/L)

FIG. 1

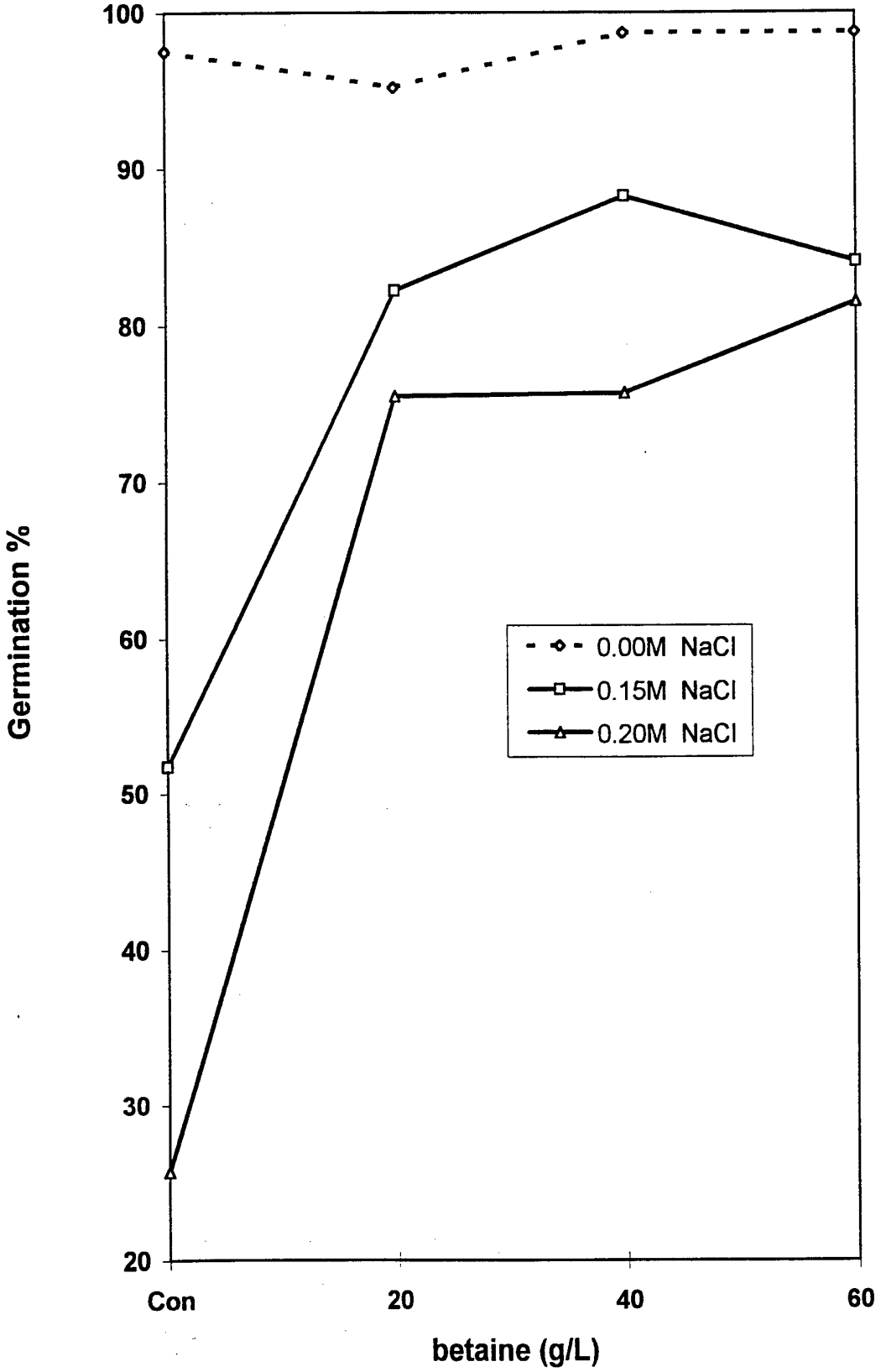
100
90
80
70
60
50
40
30
20
Germination %
0.00M NaCl
0.15M NaCl
0.20M NaCl
Con
20
40
60
betaine (g/L)

FIG. 2

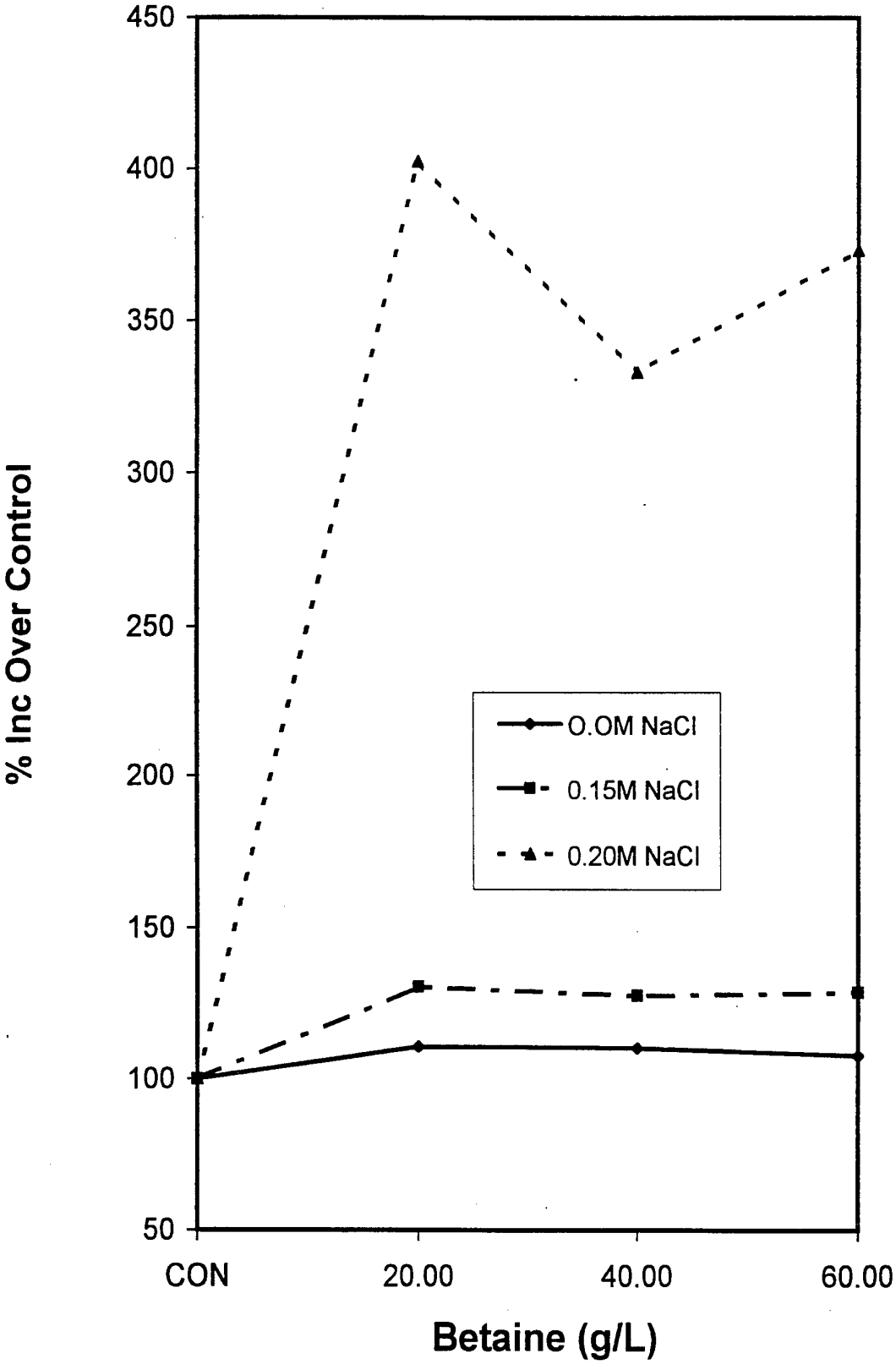
450
400
350
300
250
200
150
100
50
% Inc Over Control
0.0M NaCl
0.15M NaCl
0.20M NaCl
CON
20.00
40.00
60.00
Betaine (g/L)

FIG. 3

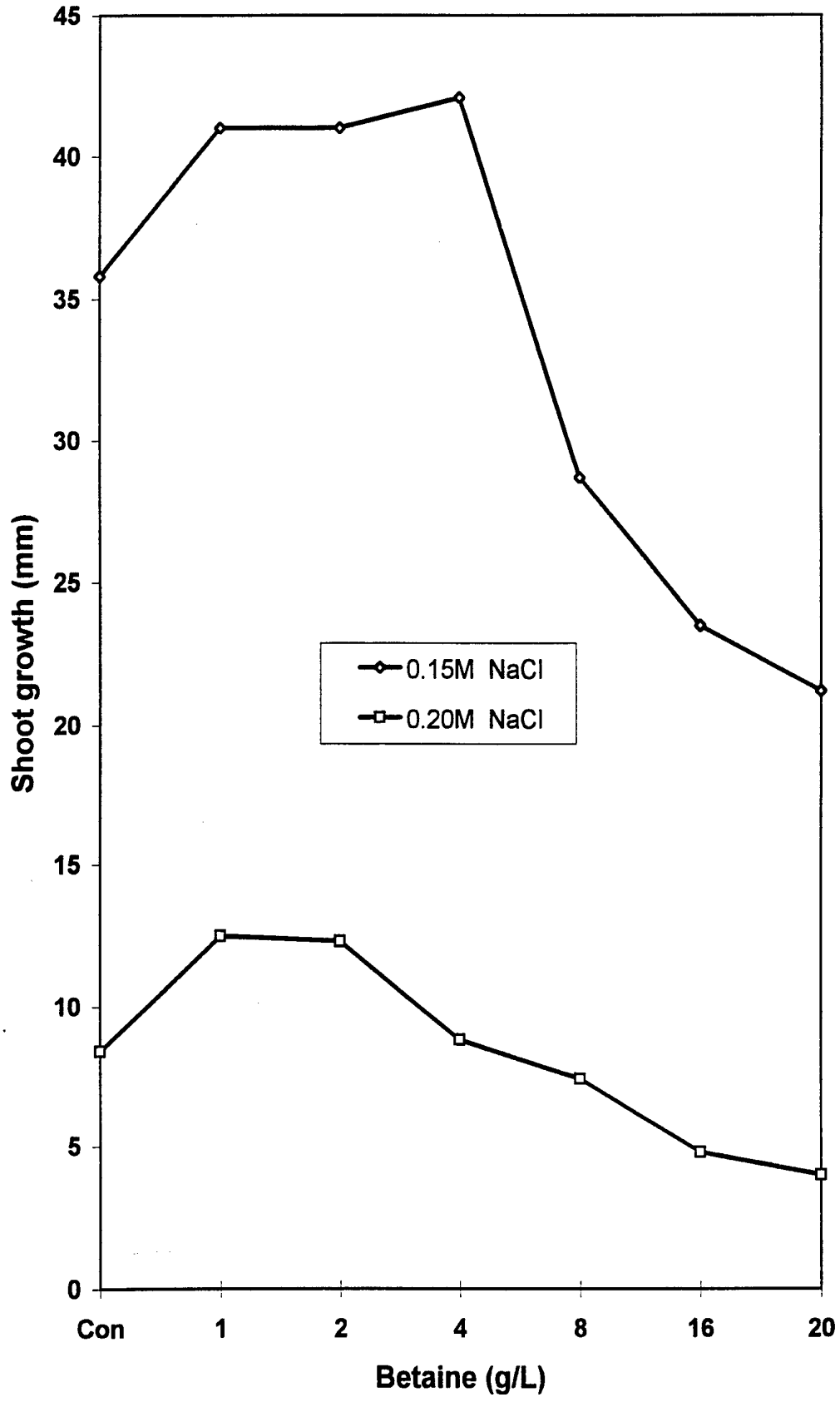
45
40
35
30
25
20
15
10
5
0
Shoot growth (mm)
0.15M NaCl
0.20M NaCl
Con
1
2
4
8
16
20
Betaine (g/L)

FIG. 4

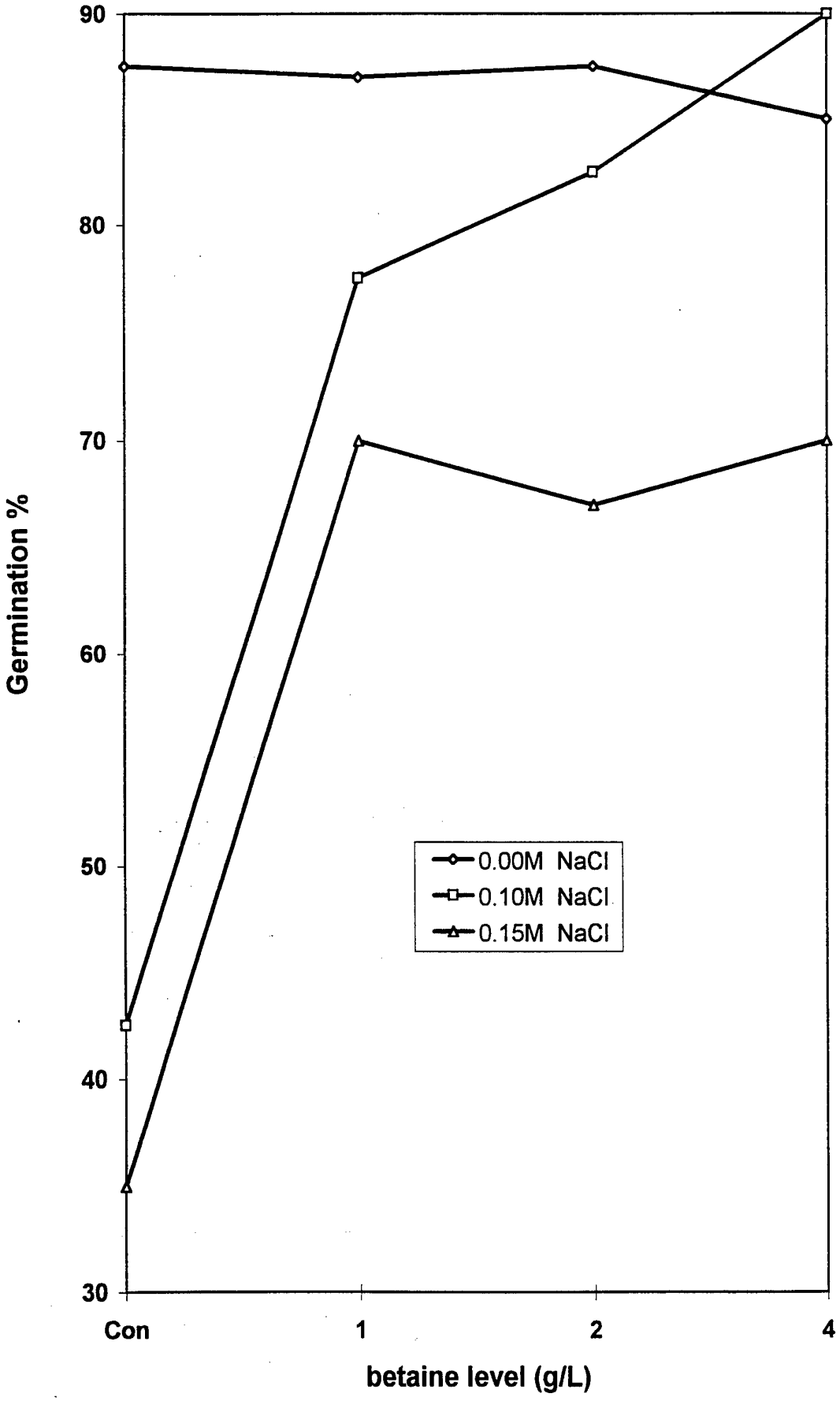
90
80
70
60
50
40
30
Germination %
0.00M NaCl
0.10M NaCl
0.15M NaCl
Con
1
2
4
betaine level (g/L)

FIG. 5

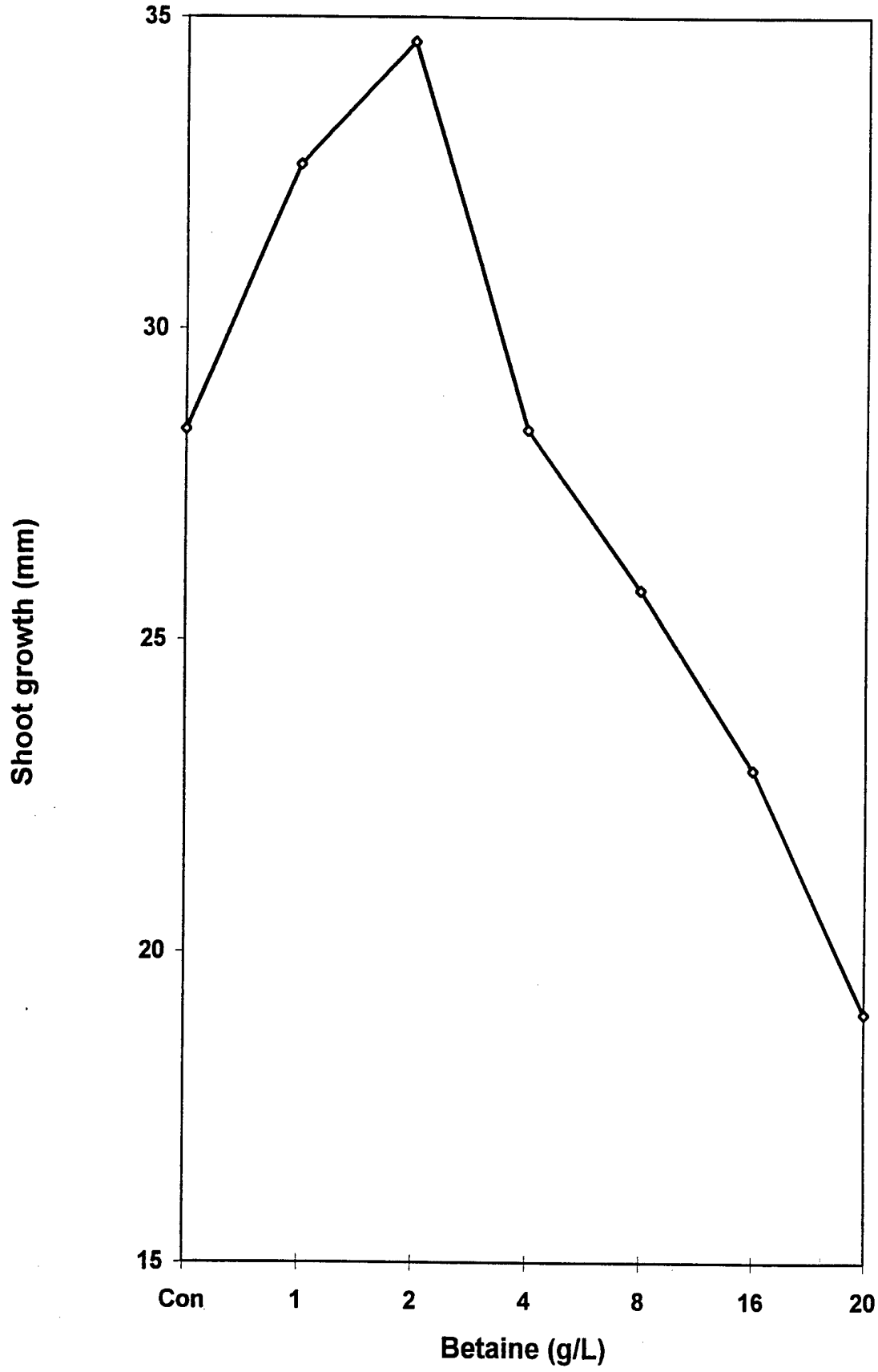

FIG. 6

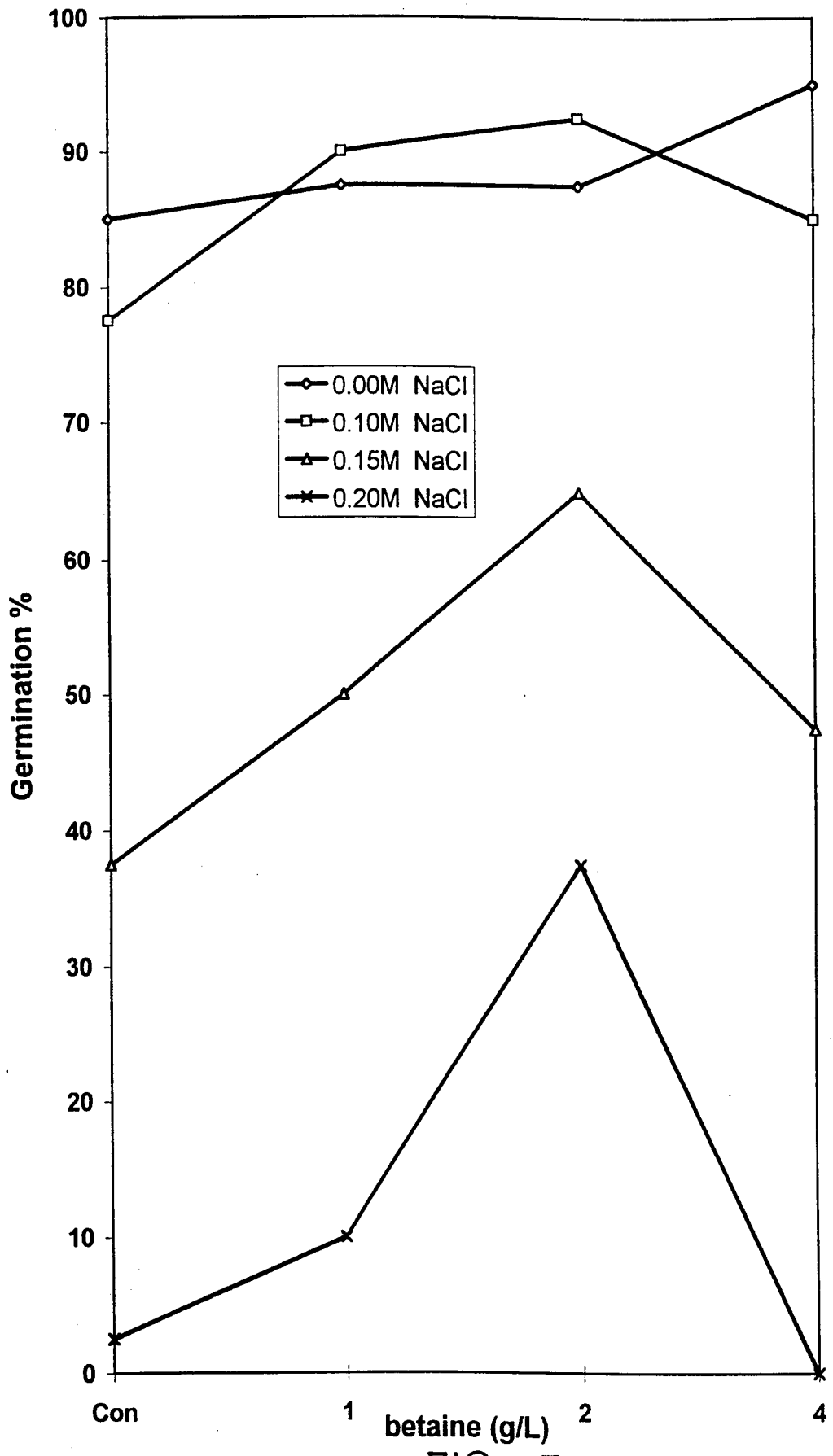
100
90
80
70
60
50
40
30
20
10
0
Germination %
0.00M NaCl
0.10M NaCl
0.15M NaCl
0.20M NaCl
Con
1
2
4
betaine (g/L)

FIG. 7

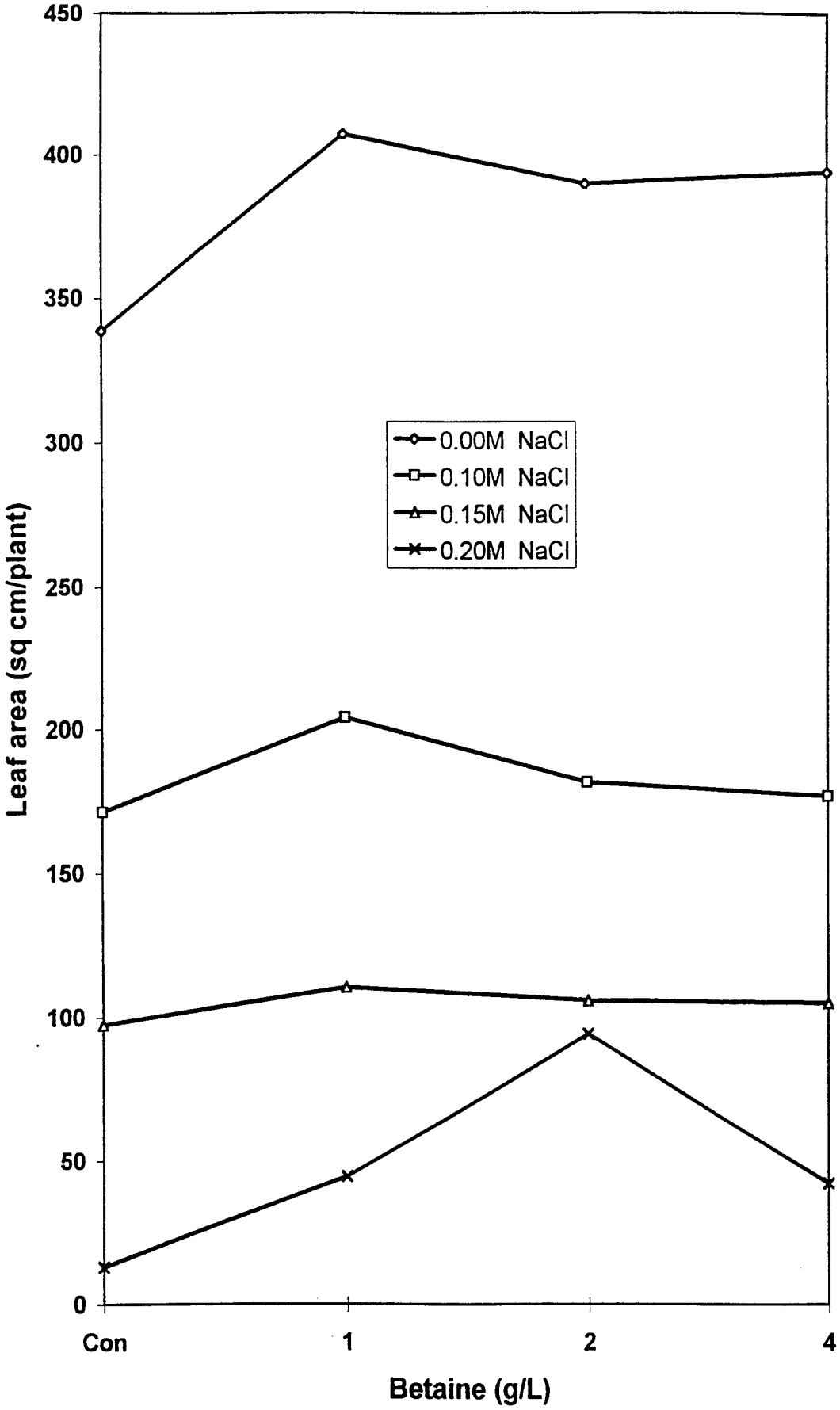
450
400
350
300
250
200
150
100
50
0
Leaf area (sq cm/plant)
0.00M NaCl
0.10M NaCl
0.15M NaCl
0.20M NaCl
Con
1
2
4
Betaine (g/L)

FIG. 8

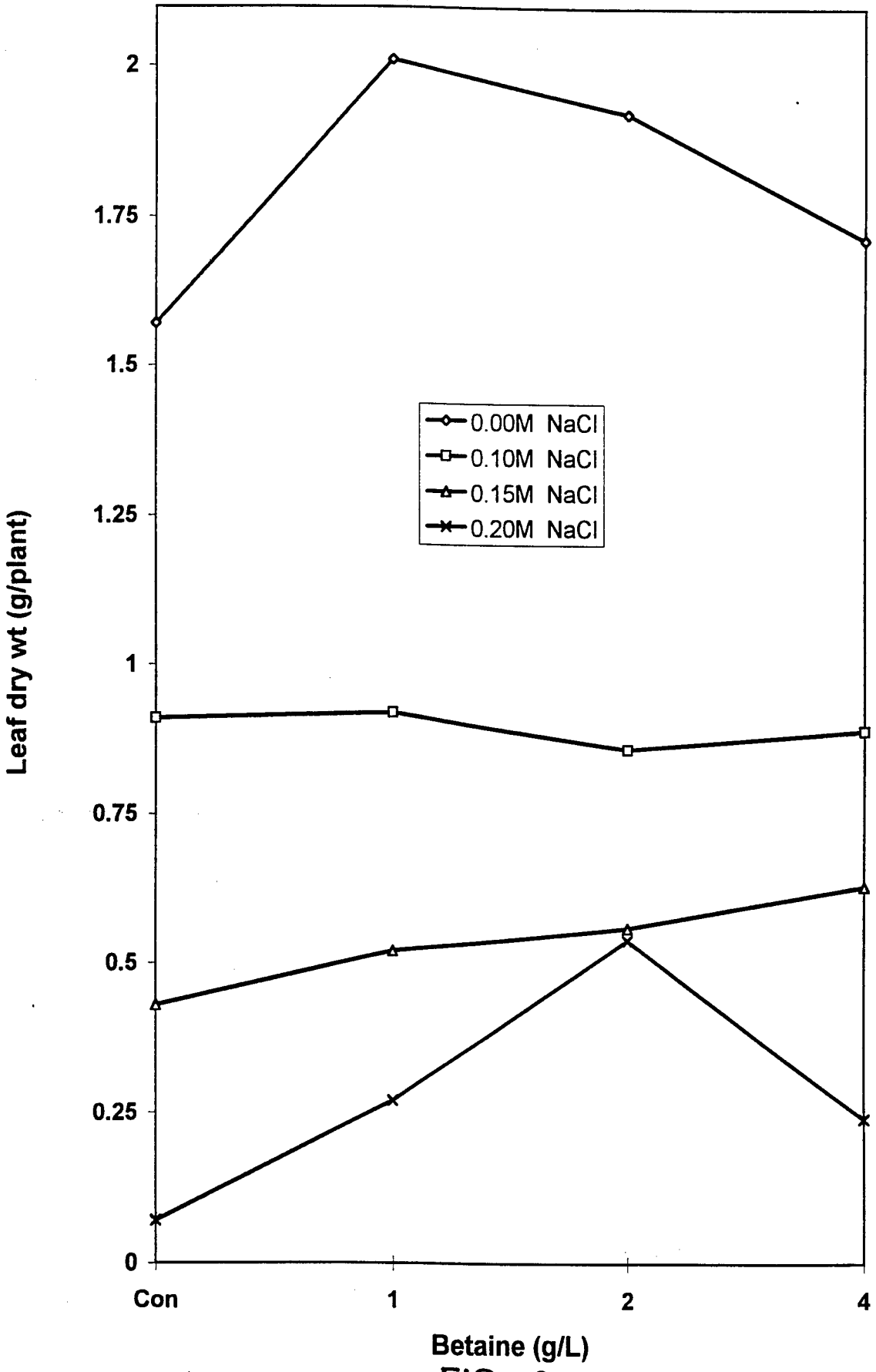
Leaf dry wt (g/plant)
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
0.00M NaCl
0.10M NaCl
0.15M NaCl
0.20M NaCl
Con
1
2
4
Betaine (g/L)

FIG. 9

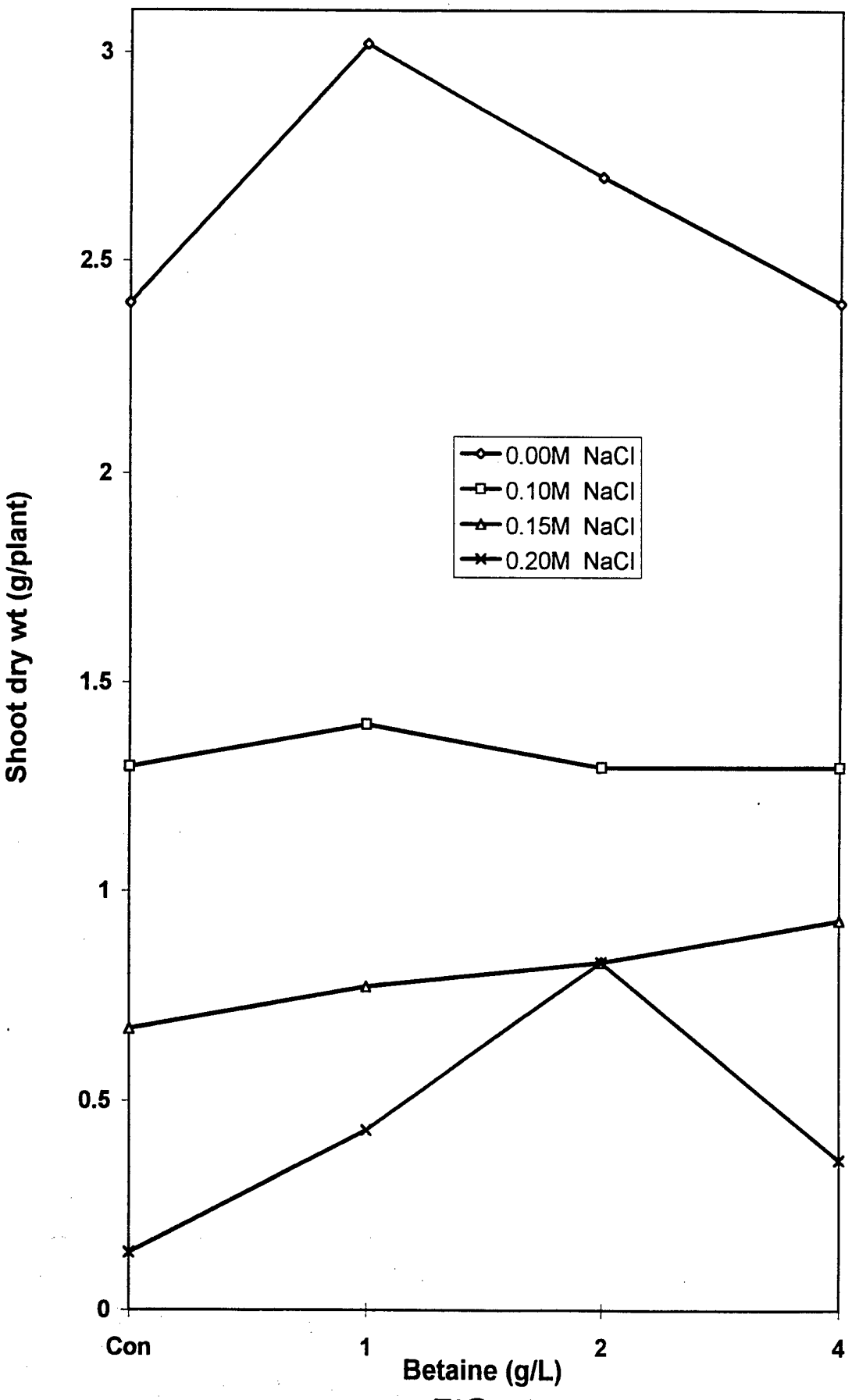
Shoot dry wt (g/plant)
3
2.5
2
1.5
1
0.5
0
0.00M NaCl
0.10M NaCl
0.15M NaCl
0.20M NaCl
Con
1
2
4
Betaine (g/L)

FIG. 10

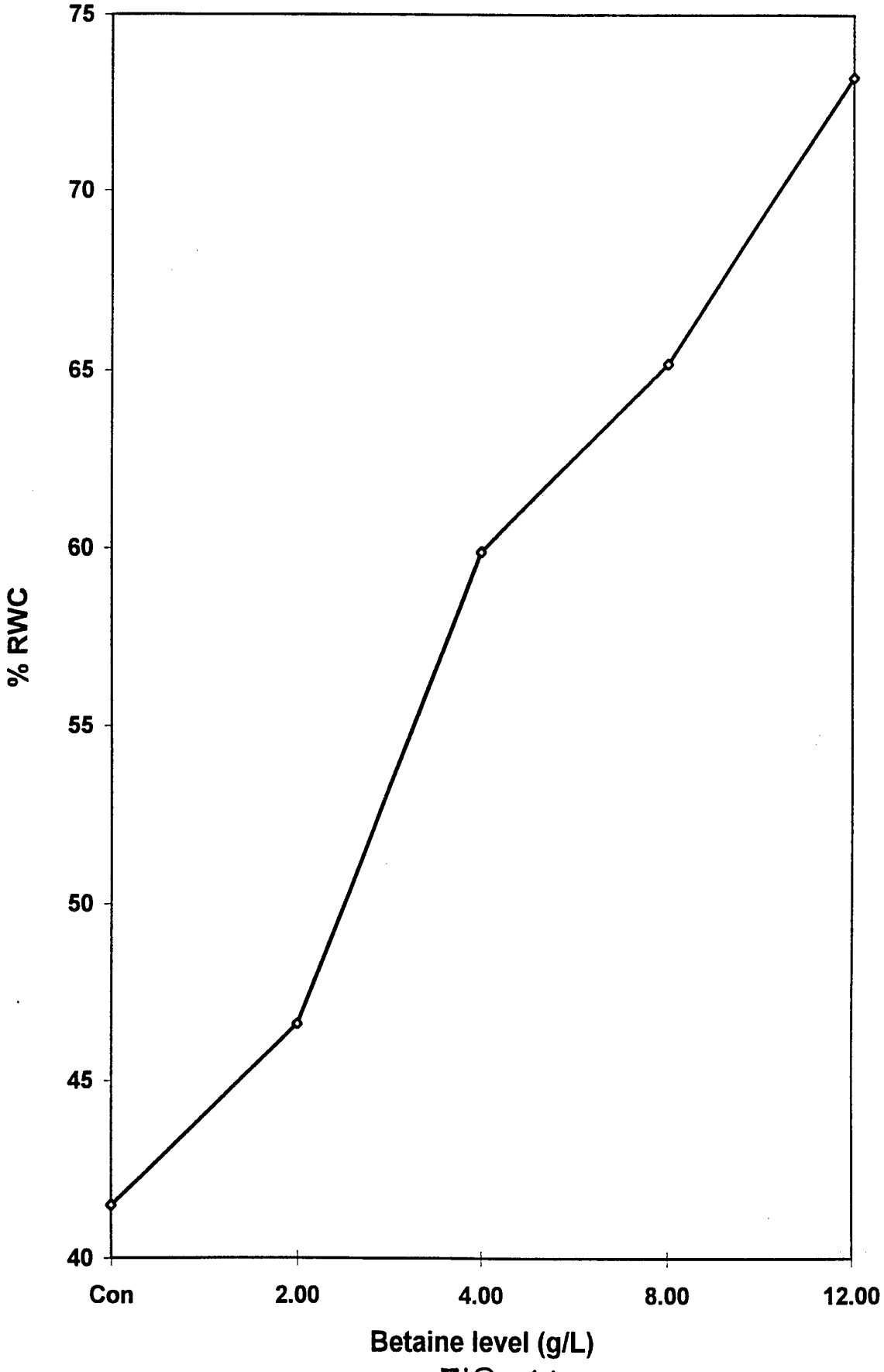

FIG. 11

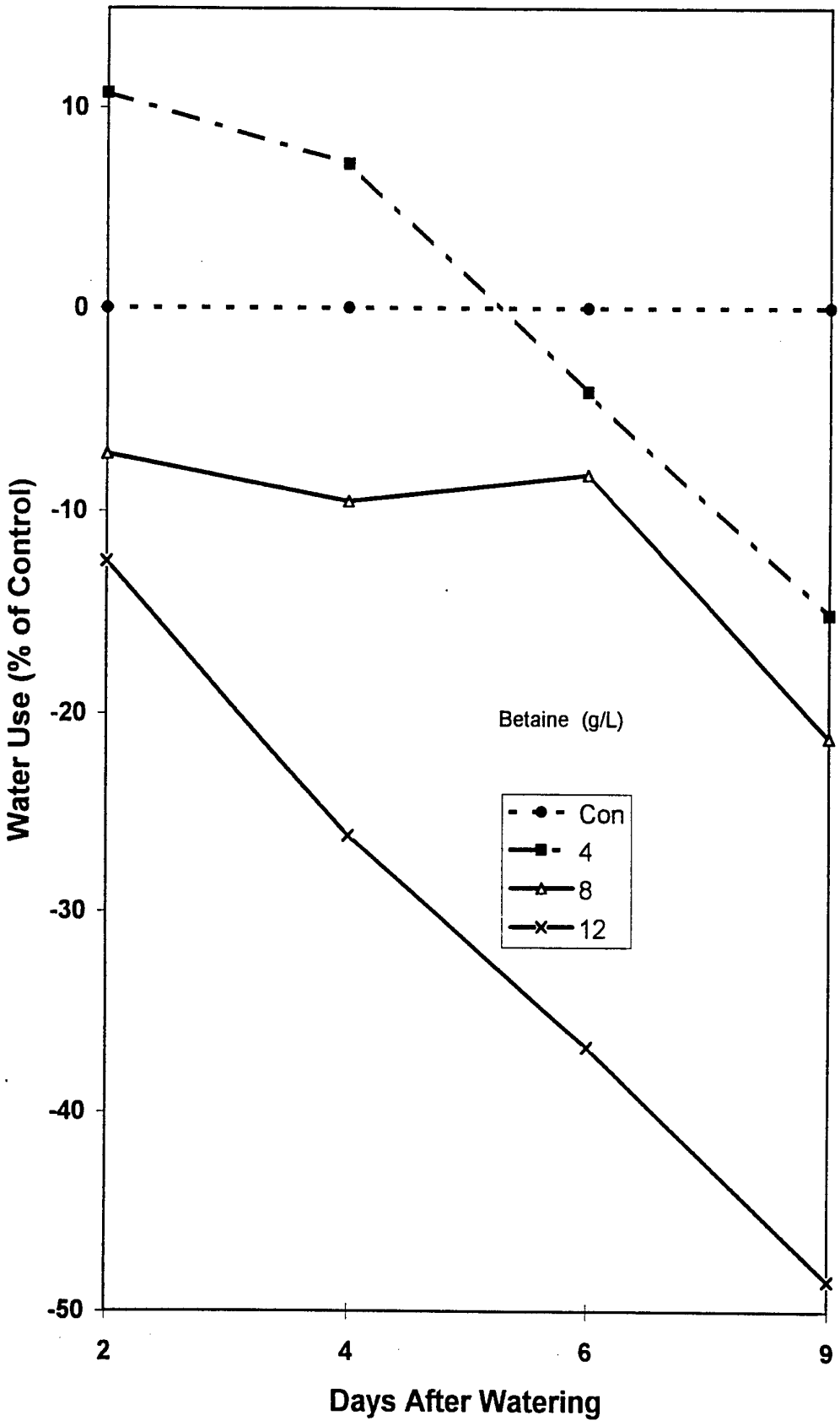
10
0
-10
-20
-30
-40
-50
Water Use (% of Control)
2
4
6
9
Days After Watering
Betaine (g/L)
Con
4
8
12

FIG. 12

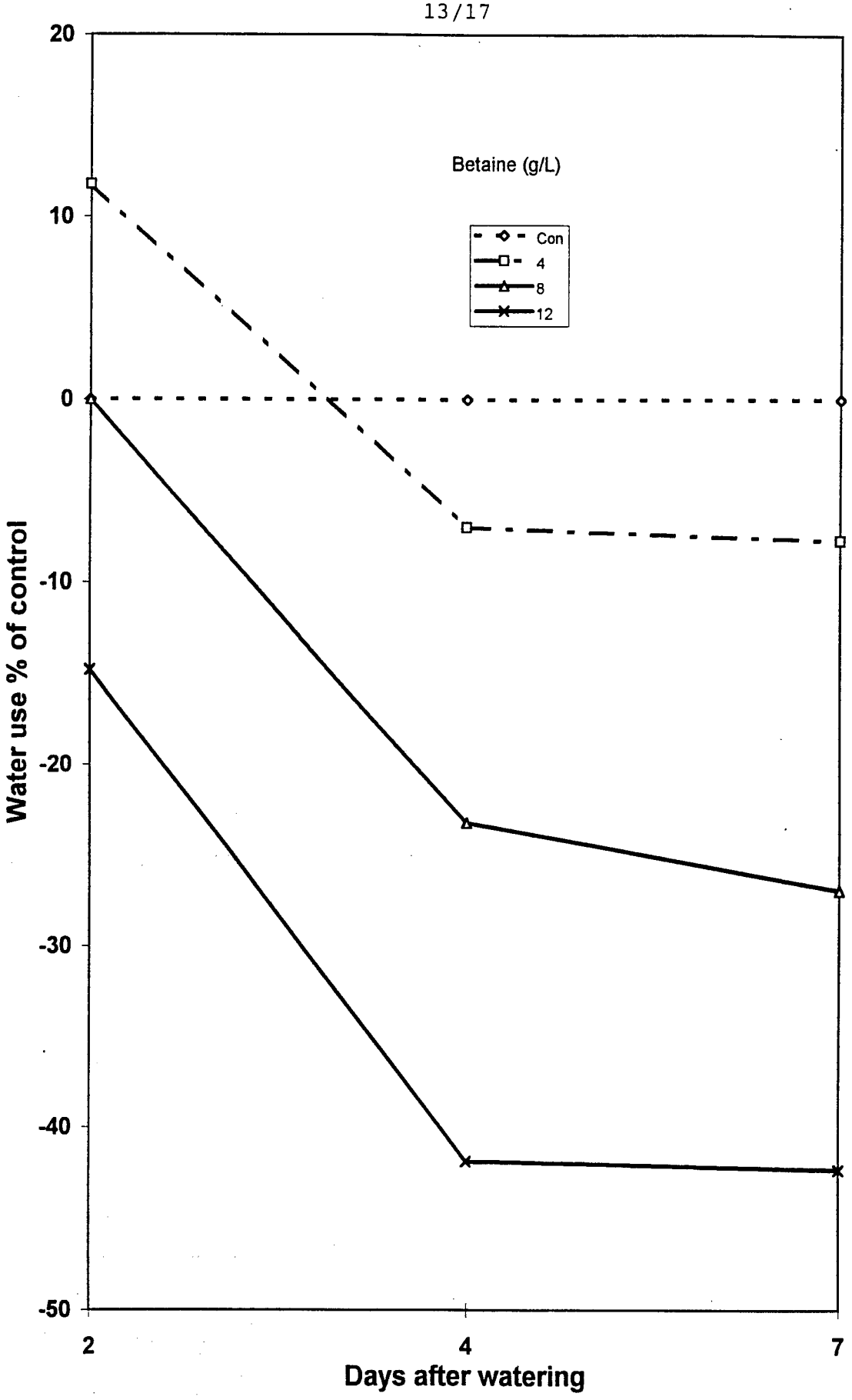
Betaine (g/L)
Con
4
8
12
Water use % of control
20
10
0
-10
-20
-30
-40
-50
2
4
7
Days after watering

FIG. 13

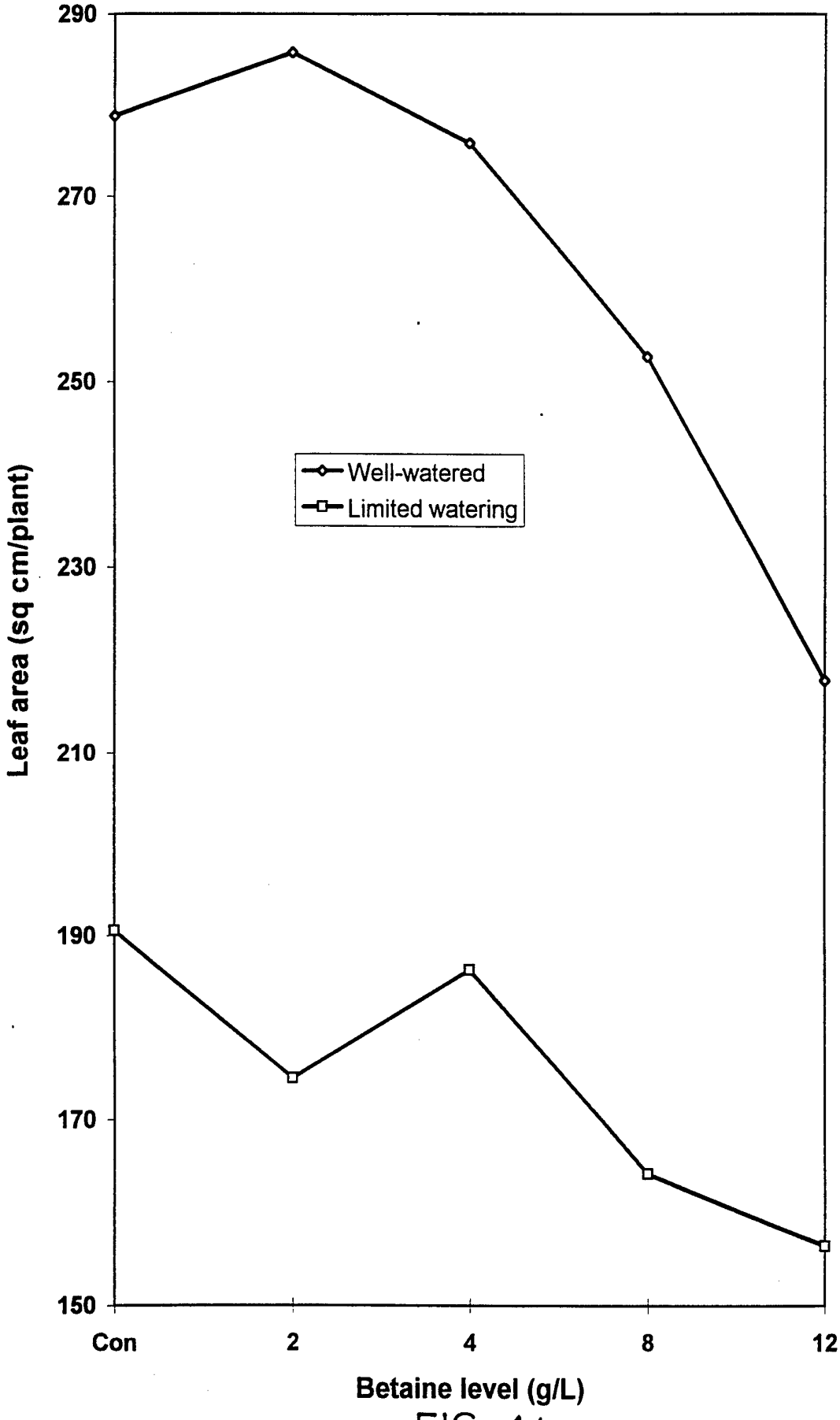
290
270
250
230
210
190
170
150
Leaf area (sq cm/plant)
Well-watered
Limited watering
Con
2
4
8
12
Betaine level (g/L)

FIG. 14

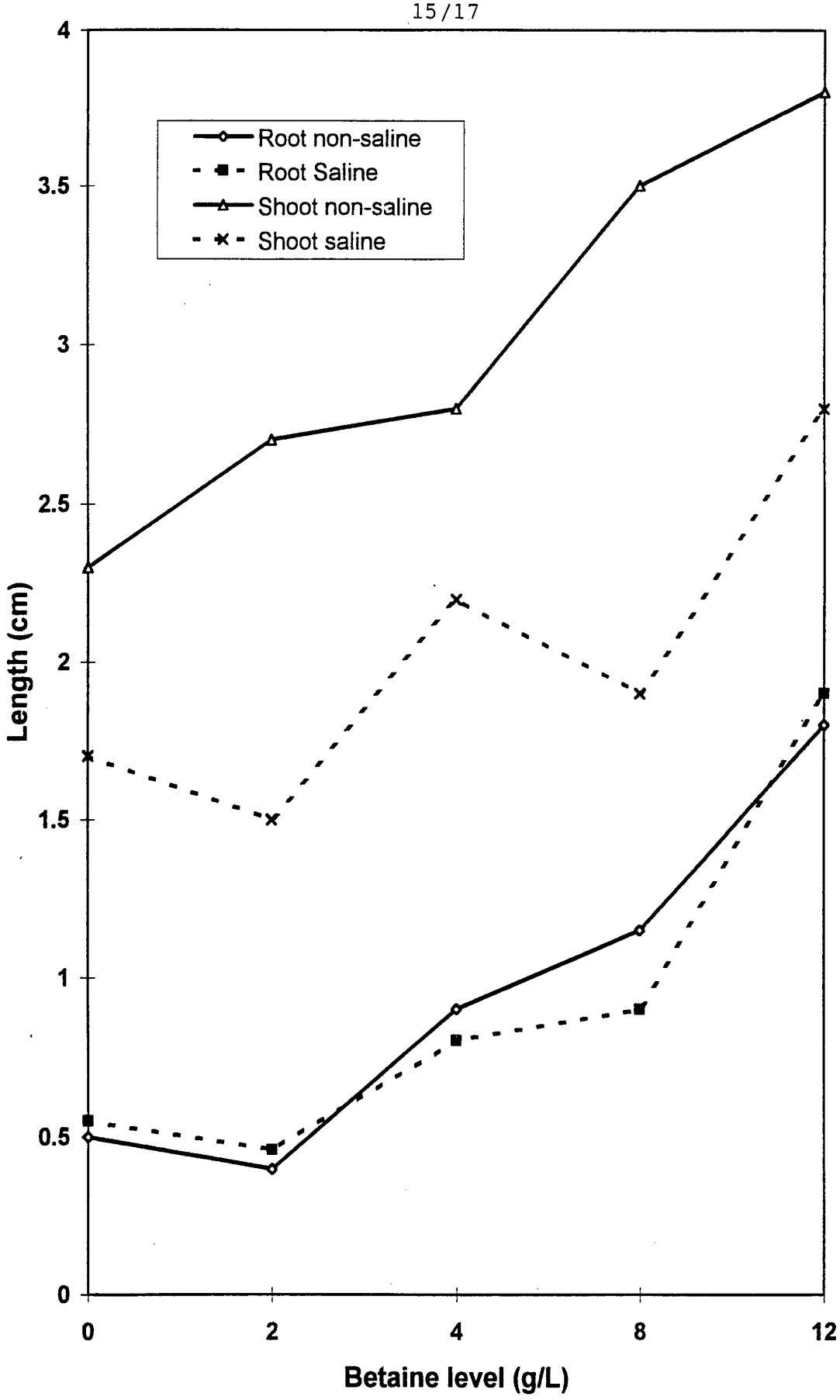
Root non-saline
Root Saline
Shoot non-saline
Shoot saline
Length (cm)
0
0.5
1
1.5
2
2.5
3
3.5
4
0
2
4
8
12
Betaine level (g/L)

FIG 15

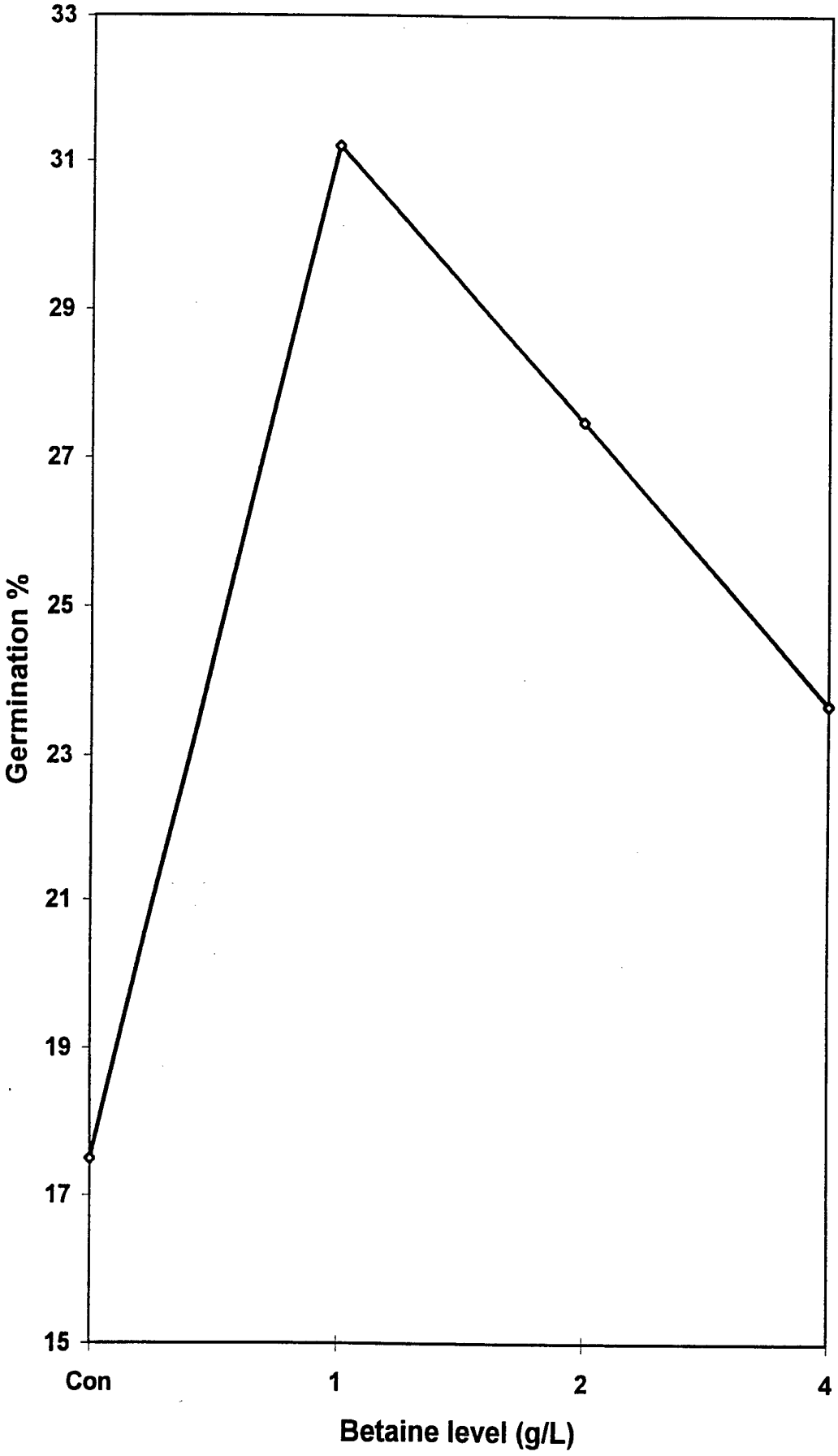
33
31
29
27
25
23
21
19
17
15
Germination %
Con
1
2
4
Betaine level (g/L)

FIG. 16

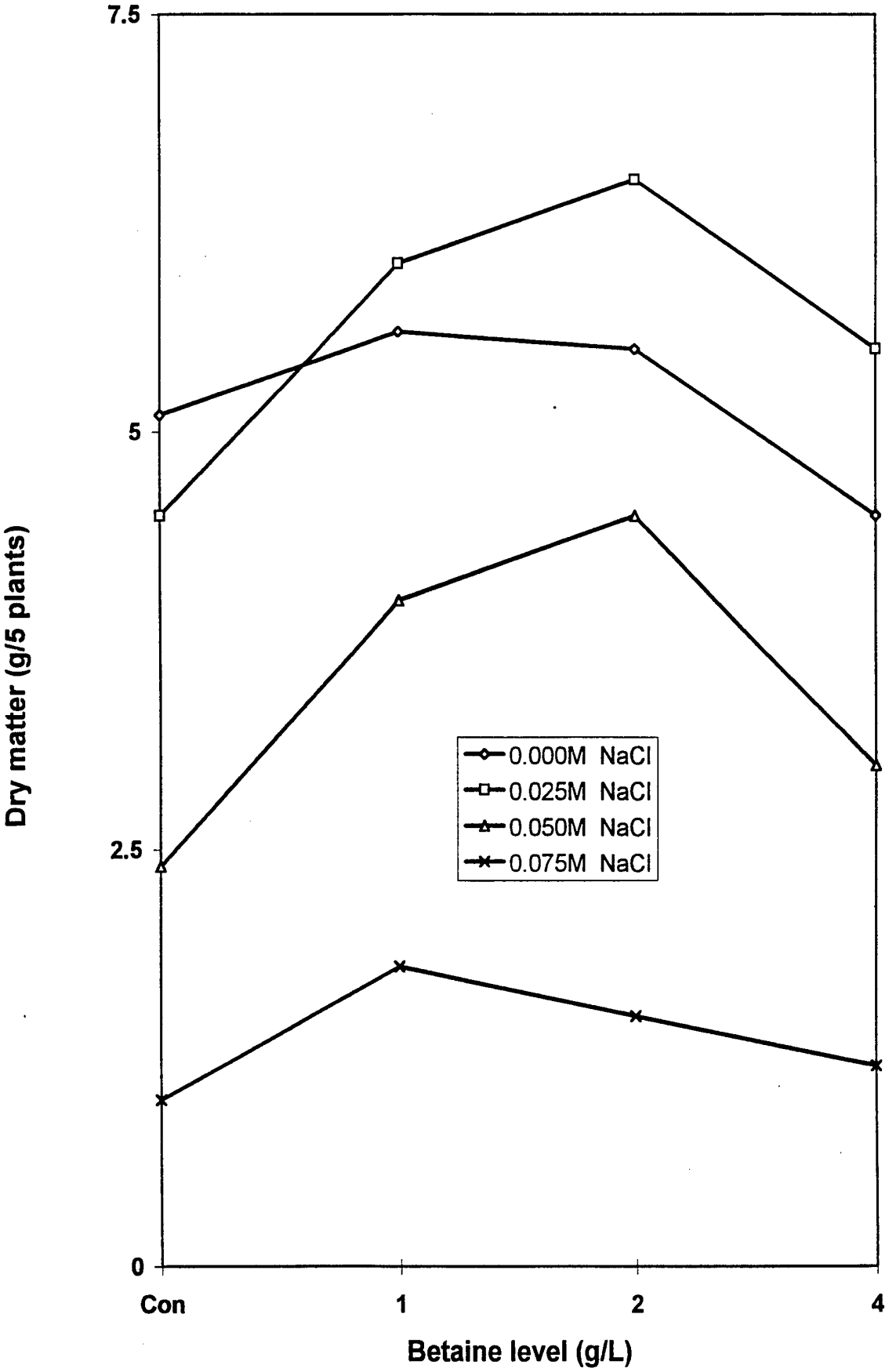
7.5
5
2.5
0
Dry matter (g/5 plants)
0.000M NaCl
0.025M NaCl
0.050M NaCl
0.075M NaCl
Con
1
2
4
Betaine level (g/L)

FIG. 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 95/00357

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl.[6] A01C 1/06

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)
IPC: A01C 1/06

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
DERWENT: KEYWORDS 'SEED:' AND "BETAINE:'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	EP 493670 A1 (BAYER AG) 8 July 1992 pages 2-5, 81-90	1-10
A	WO 8402059 A1 (MANHATTAN COLLEGE) 7 June 1984 whole document	1-10
A	AU 45058/93 A (LABORATOIRES GOEMAR S A) 20 January 1994 whole document	1

☐ 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 but 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
10 August 1995	8 SEPTEMBER 1995
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer J CARL Telephone No. (06) 2832543

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
EP	493670	DE	4038721				
WO	8402059	EP	127670				
AU	45058/93	EP	649279	FR	2693454	WO	9400993

END OF ANNEX