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Title

METHODS AND COMPOSITIONS FOR ISOLATING, IDENTIFYING AND CHARACTERIZING MONOCOT PLASTIDIC ACCASE HERBICIDE TOLERANT MUTATIONS USING A MODEL SYSTEM

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PTO/SB/16 (12-08)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PROVISIONAL APPLICATION FOR PATENT COVER SHEET – Page 1 of 2

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. _____

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Additional inventors are being named on the _____ *separately numbered sheets attached hereto.*

TITLE OF THE INVENTION (500 characters max):

METHODS AND COMPOSITIONS FOR ISOLATING, IDENTIFYING AND CHARACTERIZING MONOCOT PLASTIDIC ACCASE HERBICIDE TOLERANT MUTATIONS USING A MODEL SYSTEM

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS (*check all that apply*)

[ ] Application Data Sheet. See 37 CFR 1.76 [ ] CD(s), Number of CDs _____

[ ] Drawing(s) *Number of Sheets* 98 [ ] Other (specify) _____

[X] Specification (e.g. description of the invention) *Number of Pages* 78

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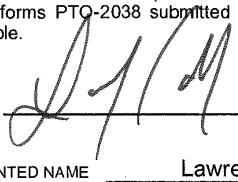
SIGNATURE  Date November 14, 2011

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METHODS AND COMPOSITIONS FOR ISOLATING, IDENTIFYING AND CHARACTERIZING MONOCOT PLASTIDIC ACCASE HERBICIDE TOLERANT MUTATIONS USING A MODEL SYSTEM

1. FIELD OF THE INVENTION

[0001] The present invention relates compositions and methods for identifying and isolating herbicide tolerant mutations in plant acetyl-CoA carboxylases.

2. BACKGROUND OF THE INVENTION

[0002] Aryloxyphenoxypropionate (FOP) and cyclohexanedione (DIM) herbicides are used post-emergence in dicot crops to control gramineous weeds. Because these herbicides effectively kill most monocotyledonous species at low concentrations, there is low toxicity to non-target organisms. Great potential exists for developing cereal varieties that can be treated post-emergence to control weedy grasses that escape other pre-emergent herbicides treatments (Somers, 1996). Furthermore, these herbicides have low persistence in soil and provide growers with increased flexibility for weed control and crop rotation. For example, red rice is the most pervasive and expensive pest in U.S. rice production (USDA-ARS Dale Bumpers National Rice Research Center 2006 Annual Report) and can serve as a host for rice diseases. CLEARFIELD® Rice is the premier tool for managing red rice in infested areas; however, gene flow between red rice and CLEARFIELD® Rice can result in ~170 F1 hybrids/ha (Shivrain et al., 2007). Thus, stewardship guidelines limit CLEARFIELD® Rice market penetration to two out of any four years on any given field. Therefore, the generation of cultivated rice with tolerance to different herbicides will provide farmers CLEARFIELD® with a rotation partner to help manage red rice weed populations.

[0003] FOPs and DIMs target the enzyme Acetyl-CoA Carboxylase (EC 6.4.1.2), which catalyzes the first committed step in fatty acid (FA) biosynthesis. ACCase is a biotinylated enzyme that converts acetyl-CoA to malonyl-CoA in a 2-step reversible reaction. The enzyme first carboxylates the biotin group and then the intrinsic carboxytranferase activity transfers the carboxyl group from carboxybiotin to acetyl-CoA (Nikolau et al., 2003). ACCase activity is necessary in the plastid which is the primary site for FA biosynthesis for membrane biogenesis. ACCase activity is also present in the cytosol, where it is involved in the synthesis of very long chain FA and flavonoids (Chugh and Eudes, 2008). The multidomain, cytoplasmic monocot and dicot ACCases are not sensitive to the FOP and DIM herbicides. It is the plastidic form of ACCase that confers the selectivity to this class of

herbicides. Dicot plastidic ACCases are naturally insensitive while monocot plastidic ACCases are herbicide-sensitive. The plastidic ACCases are highly expressed in meristematic tissue to feed the high demand for membrane biogenesis in rapidly growing, young seedlings (Podkowinski et al., 2003). This essential role for growth explains the effectiveness of targeting ACCase inhibition in post-emergent weeds.

[0004] Since the inception of FOP and DIM use for controlling weeds in world agriculture in the 1980s, there has been an emergence of tolerance amid various weed species. Among these, the most extensively studied are *Alopecurus myosuroides* (blackgrass) and *Avena sterilis* (wild oat). Comprehensive studies of natural blackgrass and wild oat mutants have revealed six residues within the carboxyltransferase domain of the plastidic isoform that confer tolerance to FOPs and/or DIMs (Delye et al., 2003; Delye et al., 2005; Liu et al., 2007) and these are I1781L, W2027C, I2041N, G2096A, D2078G and C2088R (designation according to standard blackgrass ACCase reference sequence). Interestingly, I1781L and D2078G confer tolerance to both FOPs and DIMs while the other four mutations confer tolerance only to FOPs, suggesting that the binding sites of the two classes of herbicides is overlapping, yet distinct.

[0005] Two approaches to develop DIM tolerant rice that have been tried include the following. In the first approach, previously identified mutations in natural blackgrass and wild oat are introduced by means of molecular biological techniques in rice plastidic ACCase. The effects of the mutations are studied in rice after *Agrobacterium*-mediated transformation of the modified ACCase genes. Ultimately, the same mutations can be engineered at the endogenous locus through oligonucleotide gene targeting (Beetham et al., 1999). In the second approach rice callus is propagated in medium with gradually increasing DIM concentrations. This procedure can enrich the callus for cells in which plastidic ACCase has mutated to become more tolerant to the herbicide. Plants can be regenerated from the callus when a satisfactory tolerance level has been reached. However, both approaches are limited in the number of mutations that can be generated and/or tested.

[0006] Yeast is an excellent model organism for screening and testing large numbers of mutated rice ACCase genes for increased herbicide tolerance. However, yeast contains a single, endogenous ACCase gene (ACC1), which encodes a multidomain protein that is highly tolerant to herbicides. Haploid yeast in which ACC1 is disrupted is not viable (Hasslacher et al., 1993). Joachimiak et al. (1997) introduced wheat cytoplasmic ACCase into a diploid strain heterozygous for ACC1. Standard tetrad analysis demonstrated that the

ACC1 mutation was complemented by the plant ACCase. A similar experiment with the herbicide-sensitive wheat plastidic ACCase gene showed that this gene was not able to take over the function of *ACC1* (Nikolskaya et al., 1999). A series of chimeric constructs consisting of the N-terminus of wheat cytoplasmic ACCase and the C-terminus of wheat plastidic ACCase was tested for complementation and herbicide sensitivity. Wheat c60p40 ACCase, which is comprised of the first 60% of cytoplasmic ACCase and last 40% of plastidic ACCase, complemented an ACCase deletion mutant, Δacc1, while showing the highest sensitivity towards haloxyfop, clodinafop, quizalafop, cethoxydim and sethoxydim of all constructs tested. The c60p40 chimeric ACCase is a suitable target for mutagenesis, as all herbicide-conferring mutations known have been mapped to the last 40% of plastidic ACCase. Yet, the Joachimiak approach is limited in its efficiency in that each new mutant plastid ACCase construct is separately introduced into yeast cells to create a new yeast knockout complement.

[0007] DIMs and FOPs are important herbicides and there is a need for methods and compositions to isolate, identify and characterize herbicide tolerant ACCase variants. The methods and compositions described herein are suitable for isolating, identifying, and characterizing such herbicide tolerant ACCase variants. Citation or discussion of a reference herein shall not be construed as an admission that such is prior art to the present invention.

3. SUMMARY OF THE INVENTION

[0008] In some embodiments, the present invention provides methods of producing an acetyl-CoA carboxylase (ACCase) enzyme that is tolerant to at least one herbicide. Typically, the methods of the invention are high throughput methods. Such methods will typically comprise, providing an ACCase-deficient yeast that comprises a nucleic acid encoding a chimeric ACCase. Any nucleic acid molecule known in the art may be used for this purpose. Suitable examples include, but are not limited to, plasmids, for example single copy plasmids. The chimeric ACCase encoded by the nucleic acid will comprise two or more regions. In some embodiments, the nucleic acid will comprise an N-terminal region and a C-terminal region, wherein the C-terminal region comprises an herbicide sensitivity region (HSR). The ACCase-deficient yeast comprising the nucleic acid will be contacted with at least one mutagenic oligonucleotide under conditions that permit site-directed mutagenesis of at least one codon of the nucleic acid encoding the chimeric ACCase. The mutagenized yeast can then be grown thereby forming a library of mutagenized yeast colonies. The library of

mutagenized yeast colonies can be cultured in the presence of at least one ACCase-inhibiting herbicide to form treated colonies; and at least one of said treated colonies isolated so as to identify at least one mutagenized yeast that grows in the presence of the herbicide, wherein the mutagenized yeast that grows in the presence of the herbicide comprises a mutagenized ACCase that has a tolerance to the herbicide that is greater than that exhibited by the chimeric ACCase prior to mutagenesis. As indicated above, typically these methods are high throughput, for example, in an embodiment of the present method, one trained worked can generate and screen at least 500 different HSR-variant ACCases, each from its own cell colony, in 1 month, more preferably at least or about 1000, 2000, or 4000, and preferably up to or about 5000, 8000, or 10000 per month; as compared to the Nikolskaya yeast method (tetrad dissection) in which one worker could accomplish 10 to less than about 50. In some embodiments invention, the present methods are capable of producing at least about 100, 200, 300, 400, 500, 1,000, 2,500, 5,000, or 10,000 different HSR-variant ACCases per month per trained worker. Preferably methods of the invention are capable of producing at least about 500, 1,000, 2,500, 5,000, or 10,000 different HSR-variant ACCases per month per trained worker. This assumes that the trained worker in either case begins with all needed supplies of competent cells and vectors useful to transform those cells either with mutagenic oligos according to a present embodiment or with the ACCase variant according to the Nikolskaya method; and that no cell colonies are split to form multiplicates for assaying, after the clonal colonies are established.

[0009] The present invention relates to methods of producing, isolating, identifying and characterizing herbicide tolerant ACCase variants. In one embodiment, the invention encompasses a method of screening for an acetyl-CoA carboxylase (ACCase) enzyme which is tolerant to at least one herbicide, comprising: a) providing an ACCase-deficient yeast with a chimeric ACCase, said chimeric ACCase comprising: at least two regions wherein said regions further comprise; i) an N-terminal region, said N-terminal region derived from yeast, fungi or monocot cytoplasmic ACCases, preferably a yeast ACCase; ii) a C-terminal region, said C-terminal region derived from monocot plastidic ACCases and comprising an HSR; and iii) said N-terminal region comprises about 50% to about 60% of the chimeric ACCase; b) isolating herbicide tolerant yeast cells after culturing in the presence of at least one herbicide; and c) further comprising identifying the mutation(s) not present in chimeric ACCase prior to culturing, which confers tolerance to at least one herbicide.

[0010] In other embodiments, the present invention relates to a yeast cell tolerant to at least one herbicide wherein the cell is produced by: a) complementing an ACCase-deficient yeast with a chimeric ACCase, said chimeric ACCase comprising at least two regions wherein said regions further comprise; i) an N-terminal region, said N-terminal region derived from yeast, fungi or monocot ACCases; ii) a C-terminal region, said C-terminal region derived from one or more monocot-species plastidic ACCases and comprising an HSR; and iii) said N-terminal region comprises about 50% to about 60% of the chimeric ACCase; and b) isolating herbicide tolerant yeast cells after culturing in the presence of at least one herbicide.

[0011] In yet other embodiments, the present invention relates to an isolated yeast cell comprising: a) no active genomic ACCase; b) a nucleic acid encoding a chimeric ACCase, said chimeric ACCase comprising at least two regions wherein said regions further comprise; i) an N-terminal region, said N-terminal region derived from yeast, fungi or monocot ACCases; ii) a C-terminal region, said C-terminal region derived from monocot-species plastidic ACCase and comprising an HSR; and iii) said N-terminal region comprises about 50% to about 60% of the chimeric ACCase; and c) at least one oligonucleotide that is mutagenic for the non-yeast ACCase.

[0012] In yet other embodiments, the present invention relates to a mutant ACCase which is tolerant to at least on herbicide wherein the ACCase is identified by: a) providing an ACCase-deficient yeast with a chimeric ACCase, said chimeric ACCase comprising at least two regions wherein said regions comprise; i) an N-terminal region, said N-terminal region derived from yeast, fungi or monocot ACCases; ii) a C-terminal region, said C-terminal region derived from monocot-species plastidic ACCases and comprising an HSR; and iii) said N-terminal region comprises about 50% to about 60% of the chimeric ACCase; b) isolating herbicide tolerant yeast cells after culturing in the presence of at least one herbicide; c)further comprising identifying the mutation(s) not present in chimeric ACCase prior to culturing, which confers tolerance to at least one herbicide; and d) recapitulating said mutation in a full-length monocot plastidic ACCase gene.

[0013] In yet other embodiments, the present invention relates to an isolated DNA molecule encoding a chimeric ACCase which is tolerant to at least one herbicide, said chimeric ACCase comprising at least two regions wherein said regions comprise; a) an N-terminal region, said N-terminal region derived from yeast, fungi or monocot cytoplasmic ACCases; b) a C-terminal region, said C-terminal region derived from monocot plastidic

ACCases and comprising an HSR, wherein said C-terminal region further comprises at least one mutation that confers tolerance to the herbicide ; and c) said N-terminal region comprises about 50% to about 60% of the chimeric ACCase.

[0014] In yet other embodiments, the present invention relates to an isolated chimeric ACCase which is tolerant to at least one herbicide, said chimeric ACCase comprising at least two regions wherein said regions comprise: a) an N-terminal region, said N-terminal region derived from yeast, fungi or monocot cytoplasmic ACCases; b) a C-terminal region, said C-terminal region derived from monocot plastidic ACCases and comprising an HSR; and c) said N-terminal region comprises about 50% to about 60% of the chimeric ACCase.

[0015] In other embodiments, methods and compositions of the invention encompass ACCase-deficient yeast due to a mutation of the genomic yeast ACCase gene which include a single point mutation, multiple point mutations, a partial deletion, a partial knockout, a complete deletion and a complete knockout.

[0016] In other embodiments, methods and compositions of the invention encompass an chimeric or mutant ACCase, wherein said N-terminal region is derived from a yeast or fungi genus selected from the group consisting of *Saccharomyces, Schizosaccharomyces, Candida, Ascomycetes Neurospora, Kluyveromyces, Picha, Cryptococcus, Chrysosporium, Yarrowia, Arxula,* and *Hansenula.*

[0017] In other embodiments, methods and compositions of the invention encompass a chimeric or mutant ACCase, wherein said N-terminal region or C-terminal region is derived from a monocot genus selected from the group consisting of *Saccharum*, *Poa*, *Agrostis*, *Lolium*, *Festuca*, *Zoysia*, *Cynodon*, *Stenotaphrum*, *Paspalum*, *Eremochloa*, *Axonopus*, *Bouteloua*, *Arundinaria*, *Bambusa*, *Chusquea*, *Guadua*, *Shibataea*, *Erharta*, *Leersia*, *Microlaena*, *Oryza*, *Zizania*, *Triticeae*, *Aveneae*, *Hordeum*, *Lolium*, *Digitaria*, *Cyperus*, *Kyllinga*, *Erigeron*, *Hydrocotyle*, *Kummerowia*, *Euphorbia*, and *Viola, Zea, Sorghum, Pennisetum, Panicum, Setaria, Eleusine, Ananas,* and *Musa.*

[0018] In a specific embodiments, methods and compositions of the invention encompass a chimeric ACCase, wherein said N-terminal region is derived from a cytoplasmic ACCase from an *Oryza* species.

[0019] In yet other specific embodiments, in other embodiments, methods and compositions of the invention encompass a chimeric ACCase, wherein said C-terminal region is derived from a plastidic ACCase from an *Oryza* species.

[0020] In other embodiments, methods and compositions of the invention encompass at least one herbicide that is a aryloxyphenoxyproprionate (FOP) or cyclohexadione (DIM) herbicide. In other embodiments, methods and compositions of the invention encompass a FOP herbicide selected from the group consisting of haloxyfop, cyhalofop, quizalofop, diclofop, clodinafop, fluazifop, metamifop, propaquizafop, and fenoxyprop. In other embodiments, methods and compositions of the invention encompass a DIM herbicide selected from the group consisting of alloxydim, butroxydim, clethodim, cycloxydim, tepraloxydim, sethoxydim, tralkoxydim, and profoxydim. In yet further embodiments, methods and compositions of the invention encompass at least one herbicide present at a concentration from about 0.02 μM to about 200 μM.

[0021] In other embodiments, methods and compositions of the invention encompass mutagenesis of the chimeric ACCase. In further embodiments, methods and compositions of the invention encompass mutagenesis induced by chemical agents, ultraviolet radiation, or a library of DNA oligos provided to the yeast cell.

[0022] In other embodiments, methods and compositions of the invention encompass a chimeric ACCase were C-terminal region comprises about 50% to about 60% of the chimeric ACCase.

[0023] In other embodiments, methods and compositions of the invention encompass yeast cells cultured at a temperature of about 23°C to about 30°C. In other embodiments, methods and compositions of the invention encompass yeast cells are cultured in liquid or on solid media.

[0024] In other embodiments, methods and compositions of the invention encompass a chimeric or mutant ACCase wherein said C-terminal region comprises a sequence which encodes for at least one mutation selected from the group consisting of I1781L, W1999G, I1781T, V2049F, V2075L, V2075I, D2078G, and V2098A.

4. BRIEF DESCRIPTION OF THE FIGURES

[0025] For the purpose of illustrating the invention, there are depicted in the drawings certain embodiments on the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

[0026] Figure 1A provides a schematic view of the domain structure of a monocot plastidic ACCase.

[0027] Figure 1B provides an overview of the OsJACCc60p40 chimeric protein. Unique restriction sites introduced in the corresponding DNA sequence for subcloning are indicated. Amino acids for which substitutions have been found to confer herbicide tolerance are indicated by white rectangles.

[0028] Figure 2A provides an overview of plasmid RTP3240-1; Figure 2B provides an overview of plasmid RTP4108; and Figure 2C provides the DNA sequence of RTP4108 (SEQ ID NO:13).

[0029] Figure 3A provides an overview of plasmid RTP3378; and Figure 3B provides the DNA sequence of the rice plastidic ACCase portion of plasmid RTP3378 (SEQ ID NO:14).

[0030] Figure 4 provides an overview of the p40 (40% plastidic) regions of four yeast vectors used in this work. All plasmids were identical to RTP4108 outside these regions. RLW001 and RTP4106 contain a small stretch that is optimized for expression in rice between Acc65I and XhoI.

[0031] Figure 5 presents an overview of directed *in vitro* evolution screening. Depicted in (a) is an overview of the rice chimeric ACCase present on RTP4108. Panels (b) through (e) represent plasmid-chromosome shuffling with the SGA reporter (can1Δ::LEU2-MFApr-HIS3/CAN1). Plasmids RTP4106, RTP4107 and RLW001 were also used for shuffling, (e)-(h). Panel (f) represents a library of synthetic DNA fragments with degenerate nucleotides at a desired position to mutagenize the chimeric ACCase present in the yeast cell. Panel (g) represents homologous recombination events that occur between the library of synthetic DNA fragments with the chimeric ACCase gene in the yeast cell followed by screening for mutations that confer tolerance to FOPs or DIMs along with sequencing of mutations conferring tolerance in panel (h).

[0032] Figure 6 represents an example of verifying the absence of an intact genomic copy of the Yeast *ACC1* gene (primer pairs A and B) along with the presence of the chimeric ACCase gene (primer pair C). Independent haploid colonies relying on RTP4108 for FA biosynthesis were checked by PCR using *ACC1*-specific primer set A (*ACC1*aFW and *ACC1*aRV) and set B (*ACC1*bFW and *ACC1*bRV) and OsJACCc60p40-specific primer set C (c60p40-5924FW and c60p40-6421RV). A yeast haploid with *ACC1* on chromosome 14 was used as a control.

[0033] Figure 7 represents a depiction of 'Kill curves' for RTP4108-8b-2 (indicated by 'c60p40'), RTP4107-5b-1 (indicated by 'c60p40 His-Tag'), RLW001-1a-1 (indicated by

'I1781L'), RTP4106-7a-1 (indicated by 'I1781L His-Tag') and yeast *ACC1* (indicated by '*ACC1*') with varying concentrations of the herbicides cycloxydim (7A), tepraloxydim (7B) , and haloxyfop (7C). Yeast containing a wild-type yeast *ACC1* gene were used as a tolerant control, center of each inset.

[0034] Figure 8 is a table showing frequency of mutations observed (columns) after transformation with different fragments and plating on different DIMs (rows). I1781X C100 = transformation carried out with fragments with degeneracy in the triplet corresponding to I1781 and plating on 100 µM cycloxydim. I1781X T1 = same transformation plated on 1 µM tepraloxydim, etc. Wt control = a fragment corresponding to the I1781X fragment, but with the "Wt" isoleucine-encoding triplet only. Shaded squares represent mutants that obtained the intended mutation (of which a fraction may be spontaneous as they only differed in one nucleotide from the "Wt" sequence).

[0035] Figure 9 is a table showing frequency of mutations observed (columns) after transformation with I2041X and W2027X fragments and plating on different DIMs (rows).

[0036] Figure 10 is a table showing frequency of observed double mutations (columns) after transformation of fragments (donor) to strains that have already one mutation in place (acceptor) and plating on 75 µM tepraloxydim. Shaded squares represent intended double mutants. DuplV2075 means a duplication of V2075.

[0037] Figure 11 is a table showing double mutations identified by plating single mutants and waiting for spontaneous second mutations.

[0038] Figure 12 is line graph showing the growth of yeast expressing a yeast ACC1 gene grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is µM concentration.

[0039] Figure 13 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is µM concentration.

[0040] Figure 14 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C

= cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0041] Figure 15 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0042] Figure 16 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0043] Figure 17 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0044] Figure 18 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0045] Figure 19 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0046] Figure 20 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0047] Figure 21 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0048] Figure 22 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0049] Figure 23 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0050] Figure 24 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0051] Figure 25 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0052] Figure 26 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0053] Figure 27 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth

is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0054] Figure 28 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0055] Figure 29 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0056] Figure 30 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0057] Figure 31 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0058] Figure 32 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0059] Figure 33 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C

= cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0060] Figure 34 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0061] Figure 35 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0062] Figure 36 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0063] Figure 37 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0064] Figure 38 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0065] Figure 39 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0066] Figure 40 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0067] Figure 41 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0068] Figure 42 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0069] Figure 43 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0070] Figure 44 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0071] Figure 45 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0072] Figure 46 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth

is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0073] Figure 47 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0074] Figure 48 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0075] Figure 49 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0076] Figure 50 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0077] Figure 51 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0078] Figure 52 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C

= cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0079] Figure 53 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0080] Figure 54 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0081] Figure 55 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0082] Figure 56 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0083] Figure 57 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0084] Figure 58 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0085] Figure 59 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0086] Figure 60 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0087] Figure 61 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0088] Figure 62 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0089] Figure 63 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0090] Figure 64 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0091] Figure 65 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth

is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0092] Figure 66 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0093] Figure 67 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0094] Figure 68 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0095] Figure 69 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0096] Figure 70 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0097] Figure 71 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C

= cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0098] Figure 72 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0099] Figure 73 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0100] Figure 74 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0101] Figure 75 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0102] Figure 76 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0103] Figure 77 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0104] Figure 78 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0105] Figure 79 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0106] Figure 80 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0107] Figure 81 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0108] Figure 82 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0109] Figure 83 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0110] Figure 84 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth

is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0111] Figure 85 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0112] Figure 86 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0113] Figure 87 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0114] Figure 88 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0115] Figure 89 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0116] Figure 90 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C

= cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0117] Figure 91 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0118] Figure 92 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

[0119] Figure 93 is line graph showing the growth of yeast expressing a chimeric ACCase gene of the invention grown in the presence of the indicated herbicides. The growth is measured as the OD_{600nm} (y-axis) as a function of time in days. The herbicides used are C = cycloxydim, T = tepraloxydim, and H =haloxyfop. The number is μM concentration. The mutation in the HSR is as indicated.

5. TERMINOLOGY

[0120] As used herein, "tolerant" or "herbicide-tolerant" indicates a yeast cell or strain; a plant; or a plant cell capable of growing in the presence of an amount of herbicide that normally causes growth inhibition in a non-tolerant (e.g., a wild-type) yeast cell or strain; a plant; or a plant cell.

[0121] As used herein, an ACCase is "sensitive" to a particular herbicide if the enzymatic activity of the ACCase is reduced in the presence of the herbicide when compared to the activity of the ACCase under identical conditions in the absence of the herbicide. Examples of herbicides to which an ACCase might be sensitive are set forth in Table 1.

[0122] Table 1 List of Herbicides

ACCase Inhibitor	Class	Company	Example Trade Names	Maximum Rate [g ai/ha]	Use
alloxydim	DIM	BASF	Fervin, Kusagard, NP-48Na, BAS 9021H	1000	POST
butroxydim	DIM	Syngenta	Falcon, ICI-A0500	75	POST

ACCase Inhibitor	Class	Company	Example Trade Names	Maximum Rate [g ai/ha]	Use
clethodim	DIM	Valent	Select, Prism, RE-45601	280	POST
clodinafop-propargyl	FOP	Syngenta	Discover, Topik, CGA 184 927	80	POST
cycloxydim	DIM	BASF	Focus, Laser, Stratos, BAS 517H	448	POST
cyhalofop-butyl	FOP	Dow	Clincher, XDE 537, DEH 112	310	POST
diclofop-methyl	FOP	Bayer	Hoegrass, Hoelon, Illoxan, HOE 23408	1120	POST
fenoxaprop-P-ethyl	FOP	Bayer	Super Whip, Option Super, Exel Super, HOE-46360, Aclaim, Puma S	111	POST
fluazifop-P-butyl	FOP	Syngenta	Fusilade, Fusilade 2000, Fusilade DX, ICI-A 0009, ICI-A 0005, SL-236, IH-773B, TF-1169	210	POST
haloxyfop-etotyl	FOP	Dow	Gallant, DOWCO 453EE	600	POST
haloxyfop-methyl	FOP	Dow	Verdict, DOWCO 453ME	600	POST
haloxyfop-P-methyl	FOP	Dow	Edge, DE 535	600	POST
metamifop	FOP	Dongbu	NA	201	POST
pinoxaden	DEN	Syngenta	Axial	60	POST
profoxydim	DIM	BASF	Aura, Tetris, BAS 625H	212	POST
propaquizafop	FOP	Syngenta	Agil, Shogun, Ro 17-3664	150	POST
quizalofop-P-ethyl	FOP	DuPont	Assure, Assure II, DPX-Y6202-3, Targa Super, NC-302	112	POST
quizalofop-P-tefuryl	FOP	Uniroyal	Pantera, UBI C4874	112	POST
sethoxydim	DIM	BASF	Poast, Poast Plus, NABU, Fervinal, NP-55, Sertin, BAS 562H	560	POST
tepraloxydim	DIM	BASF	BAS 620H, Aramo	60	POST
tralkoxydim	DIM	Syngenta	Achieve, Splendor, ICI-A0604	3400	POST

[0123] As used herein, "chimeric" refers to a non-naturally occurring polypeptide composed of two or more regions derived from different sources. Examples of a chimeric polypeptide include, but are not limited to, polypeptides having ACCase activity wherein the polypeptides have an N-terminal region derived from one source and a C-terminal region derived from another source. Examples of chimeric ACCases include, but are not limited to, a chimeric ACCase comprising an N-terminal region derived from a monocot cytosolic ACCase and a C-terminal region derived from a monocot plastidic ACCase, and a chimeric ACCase comprising an N-terminal region derived from a yeast ACCase and a C-terminal region derived from a monocot plastidic ACCase, e.g., a rice plastidic ACCase.

[0124] As used herein, an "ACCase-deficient yeast" is a yeast cell or strain that does not express a functional ACCase activity other than that of the chimeric ACCase of the invention. An ACCase-deficient yeast cell will typically comprise an exogenous source of ACCase activity, for example, an extra-genomic nucleic acid, e.g., a plasmid, encoding a chimeric ACCase of the invention.

[0125] As used herein, "recombinant" refers to an organism having genetic material from different sources.

[0126] As used herein, "mutagenized" refers to a segment of DNA that has been modified to contain one or more nucleotides that vary from the original DNA sequence. Any method known in the art may be used to induce mutations. Methods of inducing mutations may induce mutations in random positions in the genetic material or may induce mutations in specific locations in the genetic material (i.e., may be directed mutagenesis techniques).

[0127] As used herein, the terms "herbicide-tolerant" and "herbicide-resistant" are used interchangeably and are intended to have an equivalent meaning and an equivalent scope. Similarly, the terms "herbicide-tolerance" and "herbicide-resistance" are used interchangeably and are intended to have an equivalent meaning and an equivalent scope. Similarly, the terms "tolerant" and "resistant" are used interchangeably and are intended to have an equivalent meaning and an equivalent scope.

[0128] As used herein, the amino acid numbering, and the associated DNA sequence numbering are based on the numbering of the ACCase in *Alopercurus myosuroides* (blackgrass) (Genbank CAC84161.1) and denoted with an *(Am)*. The reference positions cited within are intended to correspond to the actual recited positional equivalent in the ACCase of *Alopercurus myosuroides*.

[0129] As used herein, and "herbicide sensitivity region" (HSR) is an amino acid sequence present in the carboxyl transferase domain of a monocot plastidic ACCase that: (1) when present in the ACCase in its wild-type amino acid sequence, permits the ACCase to be sensitive to inhibition by at least one DIM, FOP or DEN herbicide, and (2) is capable of being mutagenized to contain at least one amino acid substitution that provides to the ACCase tolerance toward at least one DIM, FOP or DEN herbicide. Minimally, the wild type HSR comprises a monocot plastidic ACCase amino acid sequence of or corresponding to residues 1781(*Am*)-2098(*Am*).

[0130] As used herein, "high throughput" means capable of producing at least about 100, 200, 300, 400, 500, 1,000, 2,500, 5,000, or 10,000 different HSR-variant ACCases per month per trained worker. Preferably methods of the invention are capable of producing at least about 500, 1,000, 2,500, 5,000, or 10,000 different HSR-variant ACCases per month per trained worker

DETAILED DESCRIPTION

[0131] The invention encompasses methods and compositions useful for isolating, identifying and characterizing mutations in ACCase that confer tolerance to at least one herbicide. For this purpose, a yeast model system was employed. The yeast model system included an ACCase-deficient yeast cell with a chimeric ACCase which comprises and HSR. The chimeric ACCase complements the ACCase-deficiency of the yeast. The methods and compositions encompassed by the invention relate to making and using such a system to isolate, identify and characterize mutations in ACCase which confer tolerance to an herbicide by screening or selection on media with herbicide present. Such isolated mutants in the ACCase that confer tolerance to the herbicide are not present prior to the screening or culturing in the presence of herbicide.

[0132] Prior art screening systems, for example that reported by Nikolskaya, were limited in the number of mutations that could be generated and screened. The present invention has overcome these limitations by expressing the chimeric ACCase of the invention from a single copy plasmid. Any mutation, either spontaneous or introduced through transformation will be identified far more efficiently. Prior art systems made use of high copy number plasmids (like pRS426 with 20 copies/haploid cell). In theory, a single plasmid with a herbicide resistance-conferring mutation could be formed in the presence of 19 or so wildtype plasmids in a cell. In subsequent rounds of cell division, daughter cells should start containing the mutated plasmid only, if the selection pressure is kept throughout. However, this does not happen, because the expression from the initial, single, mutated plasmid among the 19 wildtype plasmids is not robust enough to make it through the early stages of herbicide selection pressure. An additional difficulty of the prior art method is the failure of the prior art construct to complement efficiently in yeast at 30 degrees. It only complements efficiently at 23 degrees. The chimeric ACCases described herein are more efficient and complement at 30 degrees.

[0133] In some embodiments, the invention encompasses a method of screening for an acetyl-CoA carboxylase (ACCase) enzyme that is tolerant to at least one herbicide. Typically, such methods may include providing a yeast cell deficient in ACCase activity, i.e., an ACCase-deficient yeast cell. Also, such methods can include providing an ACCase-deficient yeast with a chimeric ACCase. The chimeric ACCase may comprise at least two or more regions. The chimeric ACCase may comprise an N-terminal region. The N-terminal region may be derived from a yeast, fungi or monocot cytoplasmic ACCase. The N-terminal region may comprise from about 10% to about 75% of the chimeric ACCase. The chimeric ACCase also comprises a C-terminal region. Typically, the C-terminal region may comprise from about 90% to about 25% of the chimeric ACCase. The C-terminal region comprises an HSR and may be derived from a monocot plastidic ACCase sensitive to at least one herbicide. Percentages herein are calculated relative to amino acids 98-2320 of a mature *Am* ACCase.

[0134] In other embodiments, the invention encompasses methods of isolating herbicide tolerant yeast cells expressing a chimeric ACCase after culturing in the presence of at least one herbicide. Typically, the chimeric ACCase in such herbicide tolerant yeast cells may harbor at least one mutation that confers the tolerance to the herbicide the yeast cell is cultured in. Also, the mutation(s) conferring tolerance to the ACCase and the yeast cell may be further identified using the yeast cells.

[0135] In other embodiments, the invention encompasses a yeast cell tolerant to at least one herbicide. Such a yeast cell may be produced by complementing an ACCase-deficient yeast with a chimeric ACCase. Typically, the chimeric ACCase may comprise at least two or more regions. The chimeric ACCase may comprise an N-terminal region. The N-terminal region may be derived from a yeast, fungi, dicot, or monocot cytoplasmic ACCase. The N-terminal region may comprise from about 10% to about 75% of the chimeric ACCase. The chimeric ACCase also comprises a C-terminal region. Typically, the C-terminal region may comprise from about 90% to about 25% of the chimeric ACCase. The C-terminal region comprises an HSR and may be derived from a monocot plastidic ACCase sensitive to at least one herbicide. Generally, the yeast cell may be cultured in the presence of at least one or more herbicides.

[0136] In still other embodiments, the invention encompasses an isolated DNA molecule encoding a chimeric ACCase which is tolerant to at least one herbicide. In some

embodiments, the DNA molecule encodes a chimeric ACCase which comprises at least two or more regions. Typically, the DNA molecule encodes a chimeric ACCase that may comprise at least one ore more N-terminal regions. Generally, the DNA molecule encodes an ACCase N-terminal region that may be derived from yeast, fungi, dicot or monocot cytoplasmic ACCases. The N-terminal region may comprise from about 10% to about 75% of the chimeric ACCase. The chimeric ACCase encoded by the DNA molecule also comprises at least one C-terminal region. The C-terminal region that may comprise from about 90% to about 25% of the chimeric ACCase. The C-terminal region will comprise an HSR and may be derived from a monocot plastidic ACCase sensitive to at least one herbicide.

[0137] In other embodiments, the invention encompasses an isolated chimeric ACCase which is tolerant to at least one herbicide. The chimeric ACCase may comprise an N-terminal region. The N-terminal region may be derived from a yeast, fungi, dicot or monocot cytoplasmic ACCase. The N-terminal region may comprise from about 10% to about 75% of the chimeric ACCase. The chimeric ACCase also comprises a C-terminal region. Typically, the C-terminal region may comprise from about 90% to about 25% of the chimeric ACCase. The C-terminal region comprises an HSR and may be derived from a monocot plastidic ACCase sensitive to at least one herbicide.

[0138] In yet other embodiments, the invention encompasses a mutant ACCase which is tolerate to at least one herbicide. Typically, the mutant ACCase is generated by inducing one or more mutations in the HSR of a chimeric ACCase. The chimeric ACCase may comprise an N-terminal region. The N-terminal region may be derived from a yeast, fungi, dicot or monocot cytoplasmic ACCase. The N-terminal region may comprise from about 10% to about 75% of the chimeric ACCase. The chimeric ACCase also comprises a C-terminal region. Typically, the C-terminal region may comprise from about 90% to about 25% of the chimeric ACCase. The C-terminal region comprises an HSR and may be derived from a monocot plastidic ACCase sensitive to at least one herbicide. Once induced, the mutation(s) in the chimeric ACCase that result in tolerance to herbicides in the yeast cell may be identified by, for example, sequencing the DNA molecule encoding the chimeric ACCase. Further, the method may comprise introducing the herbicide tolerant mutation(s) identified from the chimeric ACCase into a full-length, or other ACCase.

[0139] ACCase-deficient yeast and complementation with Chimeric ACCase

[0140] Yeast contain a single, endogenous ACCase gene (*ACC1*), which encodes a multidomain protein that is highly tolerant to herbicides. Haploid yeast in which *ACC1* is disrupted is not viable (Hasslacher et al., 1993).

[0141] In some embodiments, methods of the invention comprise ACCase-deficient yeast. An ACCase-deficient yeast may lack endogenous ACCase activity due to one or more mutations of the genomic yeast ACCase gene which may include a single point mutation, multiple point mutations, a partial deletion, a partial knockout, a complete deletion and a complete knockout. Typically, genomic yeast ACCase activity may be reduced or ablated using other molecular biology techniques such as RNAi, siRNA or antisense RNA. Such molecular biology techniques are well known in the art.

[0142] In some embodiments, the invention comprises introducing a nucleic acid molecule encoding a chimeric ACCase into a diploid yeast strain heterozygous for a functional genomic *ACC1* gene. Typically, the method includes verifying incorporation of the nucleic acid encoding the chimeric ACCase into a heterozygous strain. Generally, the method also may include inducing sporulation and isolation of haploid yeast cells containing an inactive yeast *ACC1* gene and a chimeric ACCase gene. Accordingly, the method may include verifying incorporation of the chimeric ACCase into a haploid strain with an inactivated genomic *ACC1* gene.

[0143] In an alternative embodiment, the invention comprises incorporation of a nucleic acid molecule encoding a chimeric ACCase into a homozygous strain followed by inactivation of one or both copies of the yeast *ACC1* gene. Typically, the method includes verifying incorporation of the chimeric ACCase gene into a homozygous strain. In yet further embodiments, the invention comprises inducing sporulation and isolation of haploid yeast cells containing an inactive yeast *ACC1* gene and a chimeric ACCase gene.

[0144] In other embodiments, the invention comprises a chimeric ACCase gene that complements a deficiency in the yeast *ACC1* gene. In other embodiments, complementation of the yeast *ACC1* gene deficiency occurs when yeast cells containing the chimeric ACCase are cultured at a temperature of about 23°C to about 30°C. In other embodiments, complementation of the yeast *ACC1* gene occurs when yeast cells containing the chimeric ACCase are cultured at a temperature of about 23°C, about 24°C, about 25°C, about 26°C, 27°C, about 28°C, about 29°C, or about 30°C. In yet other embodiments, complementation may occur at a permissive temperature, but not a restrictive temperature. Such permissive

temperatures may range from about 23°C to about 30°C. In other embodiments, such permissive temperatures may be about 23°C, about 24°C, about 25°C, about 26°C, 27°C, about 28°C, about 29°C, or about 30°C. In other embodiments, restrictive temperatures include but are not limited to a range from about 23°C to about 30°C. In other embodiments, such permissive temperatures may be about 23°C, about 24°C, about 25°C, about 26°C, 27°C, about 28°C, about 29°C, or about 30°C.

[0145] Chimeric ACCases

[0146] In one embodiment, the present invention encompasses chimeric ACCases and nucleic acid molecules encoding the same. Generally, at least a portion of the chimeric ACCase will comprise an amino acid sequence that is the same as, or corresponds to, that found in a monocot plastidic ACCase, for example, in *Alopecurus myosuroides* multi-functional ACCase 2 (Genbank CAC84161).

[0147] Monocot plastidic ACCases share a similar structure characterized by the occurrence and arrangement of 8 functional regions as shown in Figure 1A. The (*Am*) residue numbers for these regions are as follows (bullet points show regions falling within Region 1 or Region 5).

AmACCase Part	Start	Stop
AmACCase	1	2320
Region 1	135	643
• Region 2	135	257
• Region 3	259	492
• Region 4	530	637
Region 5	703	841
• Region 6	787	841
Region 7	842	1556
Region 8	1654	2204

[0148] The official COG designations from the "Clusters of Orthologous Groups" database administered by the US NIH National Center for Biotechnology Information, in Bethesda, MD, US and/or the pfam designations from the "Pfam Protein Families" database administered by the Wellcome Trust Sanger Institute, in Hinxton, Cambridge, UK of each of these regions is as follows.

[0149] Region 1: COG0439 (Biotin carboxylase [Lipid metabolism])

Region 1 containing Regions 2, 3, 4:

Region 2: pfam00289 (CPSase_L_chain) Carbamoyl-phosphate synthase L chain, N-terminal domain;

Region 3: pfam02786 (CPSase_L_D2) Carbamoyl-phosphate synthase L chain, ATP binding domain;

Region 4: pfam02785 (Biotin_carb_C) Biotin carboxylase C-terminal domain.

Region 5: COG0511 (Biotin carboxyl carrier protein [Lipid metabolism])

Region 5 containing Region 6

Region 6: pfam00364 (Biotin_lipoyl) Biotin-requiring enzyme.

Region 7: pfam08326 (ACC_central) Acetyl-CoA carboxylase, central region.

Region 8: pfam01039 (Carboxyl_trans) Carboxyl transferase domain (of biotin-dependent carboxylases).

[0150] The carboxyl transferase domain catalyzes the transcarboxylation from biotin to an acceptor molecule. All ACCases useful as sources of sequences for constructing chimeric ACCase enzymes for the practice of the present invention contain a set of these 8 regions, arranged in the same relative order. Thus, standardized residue numbering for all monocot plastidic ACCases, as well as for all dicot, yeast, and animal cytosolic/etc. multifunctional ACCases listed herein relies on the *Am* ACCase standard, to which they are all homologous.

[0151] Region 8 comprises the carboxyl transferase domain of the ACCase enzyme of the invention. Herbicide sensitivity has been mapped to a region termed the Herbicide Sensitivity Region (HSR) located in region 8 of monocot plastidic ACCases. All or a part of region 8 of a monocot plastidic ACCase may be used as an HSR. For example, a chimeric ACCase of the invention may comprise an HSR that starts anywhere from 1654(*Am*) to 1781(*Am*), and could end, e.g., anywhere from 2098(*Am*) to 2204(*Am*). Thus, an HSR of the invention may comprise amino acids corresponding to 1654(*Am*) to 2204(*Am*), 1654(*Am*) to 2130(*Am*), 1654(*Am*) to 2098(*Am*), 1750(*Am*) to 2204(*Am*), 1750(*Am*) to 2130(*Am*), 1750(*Am*) to 2098(*Am*), 1781(*Am*) to 2204(*Am*), 1781(*Am*) to 2130(*Am*), or 1781(*Am*) to 2098(*Am*). Examples of suitable HSR regions for use in the practice of the present invention include, but are not limited to, those in the following table.

[0152] Table of HSR sequences

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A. myosuroides    ihgsaaiasa ysrayeetft ltfvtgrtvg igaylarlgi rciqridqpi
O. sativa         ihgsaaiasa ysrayketft ltfvtgrtvg igaylarlgi rciqrldqpi
E. crus-galli     ihgsaaiasa ysrayeetft ltfvtgrtvg igaylarlgi rciqrldqpi
Set. italica      ihgsaaiasa ysrayeetft ltfvtgrtvg igaylarlgi rciqrldqpi
Sor. bicolor      ihgsaaiasa ysrayeetft ltfvtgrtvg igaylarlgi rciqrldqpi
T. aestivum       ihgsaaiasa ysrayeetft ltfvtgrtvg igaylarlgi rciqrtdqpi
Z. mays           ihgsaaiasa ysrayeetft ltfvtgrtvg igaylarlgi rciqrldqpi

A. myosuroides    iltgfsalnk llgrevyssh mqlggpkima tngvvhltvp ddlegvsnil
O. sativa         iltgysalnk llgrevyssh mqlggpkima tngvvhltvs ddlegvsnil
E. crus-galli     iltgfsalnk llgrevyssh mqlggpkima tngvvhltvs ddlegvsnil
Set. italica      iltgfsalnk llgrevyssh mqlggpkima tngvvhltvs ddlegvsnil
Sor. bicolor      iltgfsalnk llgrevyssh mqlggpkima tngvvhltvp ddlegvsnil
T. aestivum       iltgfsalnk llgrevyssh mqlggpkima tngvvhltvs ddlegvsnil
Z. mays           iltgfsalnk llgrevyssh mqlggpkima tngvvhltvp ddlegvsnil

A. myosuroides    rwlsyvpani ggplpitksl dpidrpvayi pentcdpraa isgiddsqgk
O. sativa         rwlsyvpayi ggplpvttpl dppdrpvayi penscdpraa irgvddsqgk
E. crus-galli     rwlsyvpani gghlpitkpl dppdrpvayi pentcdpraa irgvddsqgk
Set. italica      rwlsyvpani ggplpitkpl dppdrpvayi pentcdpraa irgvddsqgk
Sor. bicolor      rwlsyvpani ggplpitkpl dppdrpvayi pentcdpraa irgvddsqgk
T. aestivum       rwlsyvpani ggplpitksl dppdrpvayi pentcdpraa isgiddsqgk
Z. mays           rwlsyvpani ggplpitkpl dppdrpvayi pentcdpraa icgvddsqgk

A. myosuroides    wlggmfdkds fvetfegwak tvvtgraklg gipvgviave tqtmmqlvpa
O. sativa         wlggmfdkds fvetfegwak tvvtgraklg gipvgviave tqtmmqtipa
E. crus-galli     wlggmfdkds fvetfegwak tvvtgraklg gipvgviave tqtmmqlipa
Set. italica      wlggmfdkds fvetfegwak tvvtgraklg gipvgviave tqtmmqlipa
Sor. bicolor      wlggmfdkds fvetfegwak tvvtgraklg gipvgviave tqtmmqlvpa
T. aestivum       wlggmfdkds fvetfegwak svvtgraklg gipvgviave tqtmmqlipa
Z. mays           wlggmfdkds fvetfegwak tvvtgraklg gipvgviave tqtmmqiipa

A. myosuroides    dpgqpdsher svpragqvwf pdsatktaqa mldfnreglp lfilanwrgf
O. sativa         dpgqldsreq svpragqvwf pdsatktaqa lldfnreglp lfilanwrgf
E. crus-galli     dpgqldsher svpragqvwf pdsatktaqa lldfnreglp lfilanwrgf
Set. italica      dpgqldsher svpragqvwf pdsatktaqa lldfnreglp lfilanwrgf
Sor. bicolor      dpgqldsher svpragqvwf pdsatktaqa lldfnreglp lfilanwrgf
T. aestivum       dpgqldsher svpragqvwf pdsatktaqa mldfnreglp lfilanwrgf
Z. mays           dpgqldsher svpragqvwf pdsatktaqa lldfnreglp lfilanwrgf

A. myosuroides    sggqrdlfeg ilqagstive nlrtynqpaf vyipkaaelr ggawvvidsk
O. sativa         sggqrdlfeg ilqagstive nlrtynqpaf vyipmaaelr ggawvvvdsk
E. crus-galli     sggqrdlfeg ilqagstive nlrtynqpaf vyipmagelr ggawvvvdsk
Set. italica      sggqrdlfeg ilqagstive nlrtynqpaf vyipmagelr ggawvvvdsk
Sor. bicolor      sggqrdlfeg ilqagstive nlrtynqpaf vyipmagelr ggawvvvdsk
T. aestivum       sggqrdlfeg ilqagstive nlrtynqpaf vyipkaaelr ggawvvidsk
Z. mays           sggqrdlfeg ilqagstive nlrtynqpaf vyipmagelr ggawvvvdsk

A. myosuroides    inpdriecya ertakgnv
O. sativa         inpdriecya ertakgnv
E. crus-galli     inpdriecya ertakgnv
Set. italica      inpdriecya ertakgnv
Sor. bicolor      inpdriecya ertakgnv
T. aestivum       inpdriefya ertakgnv
Z. mays           inpdriecya ertakgnv
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[0153] These sequences are *Alopecurus myosuroides* 1781-2098 (GenBank accession number CAC84161); *Oryza sativa* 1792-2109 (GenBank accession number AAM18728);

Echinochloa crus-galli 1775-2092 (GenBank accession number ADR32358); *Setaria italica* 1780-2097 (GenBank accession number AAO62902); *Sorghum bicolor* 1785-2102 (GenBank accession number EES10506); *Triticum aestivum* 1769-2086 (GenBank accession number AAC49275); *Zea mays* 1783-2100 (GenBank accession number AAP78896).

[0154] Chimeric ACCases of the invention typically comprise at least two regions, an N-terminal region and a C-terminal region. Typically, the present invention may encompass full-length chimeric ACCases. Alternatively, the present invention may encompass chimeric ACCases with truncated N-terminal and/or C-terminal ends that retain acetyl-CoA carboxlase activity. Additionally, chimeric ACCases may include N-terminal and/or C-terminal regions that include sequences that are derived from the same sources as the remainder of the N-terminal and/or C-terminal regions. Such sequences may be incorporated for purposes of identification of mutants, purification of the ACCase, or other such functions.

[0155] N-terminal regions of chimeric ACCases

[0156] In some embodiments, the present invention comprises a chimeric ACCase with an N-terminal region derived from a yeast, fungi, dicot or monocot-cytoplasmic ACCase. In a specific embodiment, the N-terminal region of the chimeric ACCase may be derived from a rice cytoplasmic ACCase. Alternatively, the N-terminal region may be derived from *Saccharomyces.* In addition, although monocot cytoplasmic ACCases are preferred sources of N-terminal regions, other sources of N-terminal region include dicot, yeast, and animal cytoplasmic and other (e.g., endoplasmic reticulum) ACCases, examples of which are provided herein. Any ACCase that is multifunctional, homologous to/hybridizable with and within the same general size range -- e.g., 2000-2500 amino acids, or more preferably 2200-2400 amino acids, may be used as a source of the N-terminal region of a chimeric ACCase of the invention.

[0157] Typically, the N-terminal region comprises from about 10% to about 75% of the total length of the chimeric ACCase. More specifically, the N-terminal region may comprise about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, or about 75% of the total length of the chimeric ACCase.

[0158] When the N-terminal region comprises about 10% of the chimeric ACCase, the 3'-most amino acid residue of the N-terminal region may correspond to an amino acid in the vicinity of 319(*Am*). When the N-terminal region comprises about 20% of the chimeric ACCase, the 3'-most amino acid residue of the N-terminal region may correspond to an

amino acid in the vicinity of 542(*Am*). When the N-terminal region comprises about 30% of the chimeric ACCase, the 3'-most amino acid residue of the N-terminal region may correspond to an amino acid in the vicinity of 764(*Am*). In some embodiments, the N-terminal region comprises about 62% of the chimeric ACCase and the 3'-most amino acid residue of the N-terminal region may correspond to an amino acid in the vicinity of 1472(*Am*). In some embodiments, the N-terminal region comprises about 39% of the chimeric ACCase and the 3'-most amino acid residue of the N-terminal region may correspond to an amino acid in the vicinity of 969(*Am*). In some embodiments, the N-terminal region comprises about 33.5% of the chimeric ACCase and the 3'-most amino acid residue of the N-terminal region may correspond to an amino acid in the vicinity of 841(*Am*). In some embodiments, the N-terminal region comprises about 76% of the chimeric ACCase and the 3'-most amino acid residue of the N-terminal region may correspond to an amino acid in the vicinity of 1781(*Am*).

[0159] In some preferred embodiments, the N-terminal region comprises about 35% to about 76% of the length of the chimeric ACCase assuming that the total length of the chimeric ACCase is about the same as amino acids 98-2320 of a mature *Am* ACCase.

[0160] In some embodiments, the N-terminal region may comprise regions 1 to 7, 1 to 6, or 1-5 of an ACCase derived from a yeast, fungi, dicot or monocot-cytoplasmic ACCase.

[0161] In some embodiments, the N-terminal regions of the invention begin at the cytoplasmic methionine whose position corresponds to *Am* ACCase (plastidic) H98. In other embodiments, N-terminal regions may begin at other native methionines, e.g., the cytoplasmic methionine corresponding to *Am* ACCase M104 or S111. The following alignment shows the positions of other methionine residues in cytosolic ACCases that may be used as the first amino acid of the N-terminal region of the chimeric ACCases of the invention (see arrows below). In the alignment, cytosolic sequences are shown with numbering starting at their native Met-1 residues, whereas the plastidic sequences are shown without their leader peptides/chloroplast transit peptides (ctps).

```
 100        110         120
  |          |           |
HKASYQMNGILNESHNGRHAS-LSKVY   AmACCase plastidic (w/o leader/ctp)
VPGSYQMNGIINETHNGRHAS-VSKVV   Rice ACCase Plastidic (w/o leader/ctp)
MEGSYQMNGILNGMSNSRHPSSPSEVD   Rice Cytosolic (Genbank AAM18728)
MEGSYQMNGILNGMSNSRHPSSPSEVD   Rice Cytosolic
MVESDQING---RMSS---------VD   Wheat Cytosolic
mvesdqing---rmss---------vd   Wheat Cytosolic (Genbank AAA19970)
mvesdqingtpnrmss---------vd   Wheat Cytosolic (Genbank 2208491A)
↑      ↑       ↑
```

[0162] These are examples of preferred options for constructs utilizing a native Met position as the initiator. In other embodiments, an N-terminal region containing a plant ACCase leader peptide included upstream from the start of the cytoplasmic ACCase segment, i.e. added upstream of the position at which a cytoplasmic ACCase's native Met-1 occurs, may be used and the native Met-1 of the added leader peptide can serve as a native-type initiator Met.

[0163] In some embodiments, a construct encoding a synthetic Met site can be used to initiate the chimeric ACCase. The synthetic Met site could be situated anywhere upstream from the beginning of Region 1 of the ACCase, e.g., up to about 100 or 150 amino acids upstream therefrom, though preferably from about 10 or 15 to about 50 or 60 amino acids residues upstream of Region 1.

[0164] The presence of peptide sequence upstream from such a preferred start site is optional, e.g., leader peptides, ctp's, etc., but can be included, provided that it is other than a mitochondrial, plastidic, nuclear, endoplasmic reticulum, secretion, or other targeting peptide operative in the yeast host cell. Preferably, N-terminal leader peptides, particularly N-terminal targeting peptides, are absent from chimeric ACCases of the invention.

[0165] A yeast cytoplasmic ACCase can be used as an alternative source of an N-terminal sequence for the chimeric ACCase of the invention. As in the case of the monocot cytoplasmic ACCases, any native or synthetic Met site upstream of Region 1 of the yeast ACCase can be used as a native initiator Met for the construct; preferably native Met-1 of the yeast polypeptide is used in one embodiment. For example, *Saccharomyces cerevisiae* ACC1 (Genbank AAA20073) includes two native Met sites in its N-terminal region: Met-1 and Met-14, i.e. as numbered according to the S. cerevisiae sequence: MSEESLFESSPQKME. The numbering of these positions relative to the *Am* ACCase standard can vary, based on the alignment chosen, as being aligned with either H98(*Am*) and S111(*Am*), or A57(*Am*) and P70(*Am*), respectively. Yet, in any event, these two residues fall adjacent to I77(*Am*) and

D90(*Am*) when the yeast cytoplasmic and *Am* plastidic ACCase enzyme domains, *i.e.* Regions 1-8, are optimally aligned without also computationally aligning their upstream peptide sequences. See the exemplary alignments below, with numbering according to the *Am* standard above, and *S. cerevisiae* numbering below.

```
  60         70         80         90         100        110
   |          |          |          |          |          |
AGIIDLPKEGASAPDVDISHIDLPKEGASAPDVDISHGSEDHKASYQMNGILNESH     AmACC2
MSEESLFESSPQKME      MSEESLFESSPQKME      MSEESLFESSPQKME     ScACC1 1-15
  Alignment 1          Adjacence 2          Alignment 3
|              |     |              |     |              |
1             15     1             15     1             15
```

[0166] Monocot cytoplasmic and plastidic ACCases, and the yeast cytoplasmic ACCases, even though they all are single-chain (multi-functional) enzymes, do not necessarily correspond with each other or with *Am* ACCase in every position occupied by an amino acid. Alignment of these shows the presence of some gaps, insertions, etc. In addition to these differences, there is absent from the cytoplasmic forms a ctp-containing N-terminal leader peptide that is present in plastidic forms. Yet, commonly these monocot and yeast multifunctional forms are about 2000-2500 amino acids in length, typically about 2200-2400 amino acids in length, and all share the same 8-Region structural pattern.

[0167] The following table presents useful examples of cytosolic and other (e.g., endoplasmic reticulum) multi-functional ACCase sources for N-terminal regions in a chimeric ACCases of the invention. The table also shows key start and stop sites in native herbicide sensitive ACCases that correspond to those of the *Am* standard.

Source Type	Source Organism	Genbank Accession	Length (aa)	Reg.1 Start	Reg.7 Start	Reg.7 Stop	Reg.8 Start	Reg.8 Stop
Monocot	*Oryza sativa*	AAM18728	2267	39	745	1495	1599	2152
	Sorghum bicolor	EES16080	2248	39	728	1476	1580	2133
	Triticum aestivum	AAC49275	2260	30	736	1483	1587	2145
Dicot	*Arabidopsis thaliana*	AAC41645	2254	37	744	1485	1587	2139
	Glycine max	AAA75528	2261	39	746	1491	1593	2145
	Medicago sativa	AAB42144	2257	39	746	1487	1589	2141
Yeast	*Candida tropicalis*	EER36267	2274	103	822	1522	1615	2172
	Kluyveromyces lactis	CAG98063	2231	60	769	1477	1572	2128
	Neurospora crassa	EAA33781	2275	52	760	1500	1602	2153
	Pichia pastoris	CAY68084	2215	44	735	1457	1552	2110
	Saccharomyces cerevisiae	AAA20073	2237	59	768	1479	1574	2134
	Schizosaccharomyces pombe	BAA11238	2279	69	777	1516	1614	2169
	Yarrowia lipolytica	CAG82031	2266	67	776	1516	1610	2164
Animal	*Bos Taurus*	CAB56826	2346	118	819	1569	1669	2223
	Culex quinquefasciatus	EDS45294	2311	93	789	1531	1631	2185
	Drosophila melanogaster	AAF59156	2323	101	797	1541	1641	2196
	Gallus gallus	AAA48701	2324	118	819	1546	1646	2200
	Ovis aries	CAA56352	2346	118	819	1569	1669	2223

[0168] These sequences are preferably used without any N-terminal signal peptide segment thereof, if any is present in the native sequence. N-terminal sequences in general are optional in a chimeric ACCase of the invention, but typically about 10-50 amino acids of N-terminal sequence can be present in a chimeric ACCase of the invention. In those embodiments in which an N-terminal sequence, i.e. upstream from Region 1, is used, it is preferably devoid of any signal peptide operable in the host cell of the yeast system.

[0169] Where a yeast ACCase is used as a source of sequence for the N-terminal region of a chimeric ACCase of the invention, it will preferably be selected from a yeast species different from the species of the yeast host cell in which it is to be used. In various embodiments, monocot cytoplasmic ACCases are more preferred sources of N-terminal sequence for the constructs.

[0170] C-terminal regions of chimeric ACCases

[0171] Because the "cytoplasmic" and plastidic ACCases used as sequence sources for chimeric ACCases of the invention are homologous, the determination of the N-terminus (start point) amino acid residue of a plastidic ACCase portion, i.e., the C-terminal region, to

use in a construct generally determines the immediately upstream amino acid residue as the C-terminus of a N-terminal region, although judgment of the skilled artisan can be used to vary the selection of the actual splice point.

[0172] In some embodiments, the present invention encompasses a chimeric ACCase with a C-terminal region derived from a monocot plastidic ACCase that is sensitive to at least one herbicide. Typically, the C-terminal region confers herbicide sensitivity to the chimeric ACCase. In a specific embodiment, the C-terminal region may be derived from the rice plastidic ACCase gene.

[0173] A number of suitable monocot plastidic ACCases are identified in the table below that can serve as the source for the C-terminal region of the chimeric ACCases of the invention. The sequences of these ACCases are homologous to one another and are representative of multi-functional (single-chain) ACCases. The table also shows key start and stop sites in native herbicide sensitive ACCases that correspond to those of the *Am* standard listed in the first row.

Source Organism	Genbank Accession	Length (aa)	Reg.1 Start	Reg.7 Start	Reg.7 Stop	Reg.8 Start	Reg.8 Stop	'HSR' From	'HSR' To
Alopecurus myosuroides	CAC84161	2320	135	842	1556	1654	2204	1781	2098
Oryza sativa	EAY97401	2327	135	842	1561	1665	2216	1792	2109
Echinochloa crus-galli	ADR32358	2316	132	839	1549	1648	2201	1775	2092
Setaria italica	AAO62902	2321	132	839	1549	1653	2206	1780	2097
Sorghum bicolor	EES10506	2326	132	839	1554	1658	2211	1785	2102
Zea mays	AAP78896	2324	132	839	1552	1656	2219	1783	2100

[0174] The HSR ("Herbicide Sensitivity Region") in the above ACCases is the segment corresponding to that spanning the set of all ACCase herbicide tolerance mutations known to date, which comprises the sequence from and to the positions indicated above in the final two columns. Note that the complete HSR may include some amount of sequence further upstream and/or further downstream from the above-indicated sequence of the HSR, e.g., from about 10, 20, or 30 residues upstream and/or to about 10, 20, or 30 residues downstream of the indicated sequence, or from about 1750(*Am*), 1760(*Am*), 1770(*Am*), or 1780(*Am*) to about 2100(*Am*), 2110(*Am*), 2120(*Am*), or 2130(*Am*).

[0175] Because the HSR has now been defined to be a finite portion of Region 8 of such multi-functional ACCases, this also now make possible yeast system embodiments that employ a chimeric ACCase that comprises three regions, an N-terminal region derived from a

cytoplasmic ACCase, an HSR containing region derived from a monocot plastidic ACCase and an additional region 3' to the HSR derived from a cytoplasmic ACCase. Thus, the above-described HSR segment, or the larger Region 8 segment, or the entirety of the Region 8 can be used.

[0176] In other embodiments, the C-terminal region comprises about 50% to about 60% of the total length of the chimeric ACCase. In specific embodiments, the C-terminal region comprises about 50%, about 51%, about 52%, about 53%, about 54%, about 55%, about 56%, about 57%, about 58%, about 59%, or about 60% of the total length of the chimeric ACCase.

[0177] The native monocot ACCase HSR has been found, in the present work, to include the ACCase segment from 1781(*Am*) to 2098(*Am*). In some embodiments, a larger portion of Region 8 of an herbicide-sensitive, monocot ACCase can be used, e.g., a region starting at about 1750(*Am*), 1760(*Am*), 1770(*Am*), or 1780(*Am*), and extending to about 2100(*Am*), 2110(*Am*), 2120(*Am*), or 2130(*Am*). In some embodiments, such a later portion of an herbicide-susceptible, monocot plastidic ACCase Region 8 can comprise: the entirety of the herbicide susceptible, monocot ACCase's sequence corresponding to the segment from about 1750(*Am*) to about 2130(*Am*); or a complete herbicide-susceptible, monocot plastidic ACCase's Region 8 sequence, i.e. the sequence corresponding to the segment from about 1654(*Am*) to 2204(*Am*).

[0178] Herbicide sensitive residue positions include the following, listed with exemplary, native-type herbicide-sensitive amino acid residues: I1781(*Am*), A1785(*Am*), A1786(*Am*), I1811(*Am*), Q1824(*Am*), V1864(*Am*), W1999(*Am*), W2027(*Am*), E2039(*Am*), I2041(*Am*), V2049(*Am*), A2059(*Am*), W2074(*Am*), V2075(*Am*), D2078(*Am*), S2079(*Am*), K2080(*Am*), I2081(*Am*), C2088(*Am*), K2095(*Am*), G2096(*Am*), and V2098(*Am*). In some embodiments, one or more of these positions may be mutated to an herbicide tolerant residue and the resulting HSR included in a chimeric ACCase of the invention. Such a chimeric ACCase can be used to generate additional mutations in the chimeric ACCase that may allow for a greater herbicide tolerance than that exhibited by the chimeric ACCase having only a single herbicide tolerance mutation.

[0179] The following table provides some examples of mutations and expected herbicide tolerance characteristics of and ACCase having the indicated mutation:

Mutation	Cycloxydim Tolerance	Tepraloxydim Tolerance	Haloxyfop Tolerance
I1781L	High	Low	High
I1781T	Medium	None	Low
I1781V	Medium	Low	Medium
G1783C	Low	None	Low
A1785G	Low	None	Low
A1786P	Low	None	Medium
I1811N	Low	None	None
A1837V*	Higher	Higher	Higher
V1864F*	Lower	Higher	None
W1999C*	Higher	Higher	Higher
W1999G	High	Medium	Very High
I2041V*	Higher	Higher	Higher
V2049C*	Higher	Higher	None
V2049F	Low	Medium	None
V2049I*	Same	Higher	Same
V2049T*	Lower	Higher	None
W2074L	Low	None	None
V2075G	Low	None	Medium
V2075I	None	Low	None
V2075L	Low	High	Low
V2075M	None	Very High	Medium
2xV2075*	Same	Higher	Same
D2078G	High	High	High
D2078T	High	High	High
K2080E*	None	Higher	Higher
ΔK2080ΔI2081	None	Low	Low
C2088G*	Lower	Higher	Higher
C2088H*	Higher	Higher	Higher
C2088K*	Higher	Same	Same
C2088L*	Higher	Higher	Higher
C2088R	High	Medium	Very High
C2088T	Lower	Higher	None
C2088V*	Lower	Higher	Higher
C2088W	Low	None	None
K2095E*	None	Higher	Same
G2096A	High	Medium	High
G2096S	Medium	Low	Medium
V2098A	High	High	Medium
V2098C*	Lower	Higher	None
V2098G	High	High	Low
V2098H	Medium	Medium	Low
V2098P	Medium	High	Medium
V2098S	Medium	High	Low

*-indicates identified in double mutants

[0180] Yeast/Fungi sources of cytoplasmic ACCase

[0181] In one embodiment, the present invention encompasses cytoplasmic ACCases or portions thereof from Yeast or Fungi. Members of the Yeast/Fungi group include, but are not limited to genus members of *Saccharomyces, Schizosaccharomyces, Candida, Ascomycetes Neurospora, Kluyveromyces, Picha, Cryptococcus, Chrysosporium, Yarrowia, Arxula,* and *Hansenula.*

[0182] In other embodiments, the invention encompasses chimeric ACCases that have at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to a particular amino acid sequence for a cytoplasmic ACCases from Yeast or Fungi referenced herein.

[0183] Monocot sources of ACCase

[0184] In some embodiments, the invention encompasses cytoplasmic and/or plastidic ACCases or portions thereof from the monocot family of plants.

[0185] In another embodiment, the present invention encompasses cytoplasmic and/or plastidic ACCases of the Ehrhartoideae subfamily. Examples of the subfamily Ehrhartoideae include, but are not limited to, those of the genera *Erharta*, *Leersia*, *Microlaena*, *Oryza*, and *Zizania.*

[0186] In yet another embodiment, the present invention encompasses cytoplasmic and/or plastidic ACCases of rice plants or portions thereof. Two species of rice are most frequently cultivated, *Oryza sativa* and *Oryza glaberrima.* Numerous subspecies of *Oryza sativa* are commercially important including, but not limited to *Oryza sativa* subsp. *indica*, *Oryza sativa* subsp. *japonica*, *Oryza sativa* subsp. *javanica*, *Oryza sativa* subsp. *glutinosa* (glutinous rice), *Oryza sativa* Aromatica group (basmati), and *Oryza sativa* Floating rice group.

[0187] In one embodiment, the present invention encompasses cytoplasmic and/or plastidic ACCases of the grass family Poaceae or portions thereof. The family Poaceae may be divided into two major clades, the clade containing the subfamilies Bambusoideae, Ehrhartoideae, and Pooideae (the BEP clade) and the clade containing the subfamilies Panicoideae, Arundinoideae, Chloridoideae, Centothecoideae, Micrairoideae, Aristidoideae, and Danthonioideae (the PACCaseMAD clade).

[0188] In another embodiment, the present invention encompasses cytoplasmic and/or plastidic ACCases of the commercially important monocots or portions thereof, including, but not limited to Sugarcane (*Saccharum spp.*), as well as Turfgrasses, e.g., *Poa pratensis*

(Bluegrass), *Agrostis spp.* (Bentgrass), *Lolium spp.* (Ryegrasses), *Festuca spp.* (Fescues), *Zoysia spp.* (Zoysia grass), *Cynodon spp.* (Bermudagrass), *Stenotaphrum secundatum* (St. Augustine grass), *Paspalum spp.* (Bahiagrass), *Eremochloa ophiuroides* (Centipedegrass), *Axonopus spp.* (Carpetgrass), *Bouteloua dactyloides* (Buffalograss), and *Bouteloua var. spp.* (Grama grass).

[0189] In another embodiment, the present invention encompasses cytoplasmic and/or plastidic ACCases of the Bambusoideae subfamily or portions thereof. Examples of the subfamily Bambusoideae include, but are not limited to, those of the genera *Arundinaria*, *Bambusa*, *Chusquea*, *Guadua*, and *Shibataea*.

[0190] In another embodiment, the present invention encompasses cytoplasmic and/or plastidic ACCases of the Pooideae subfamily or portions thereof. Examples of the subfamily Ehrhartoideae include, but are not limited to, those of the genera *Triticeae*, *Aveneae*, and *Poeae*.

[0191] In yet another embodiment, the present invention encompasses cytoplasmic and/or plastidic ACCases of wheat plants or portions thereof. Two species of wheat are most frequently cultivated, *Triticum aestivum, and Triticum turgidum.* Numerous other species are commercially important including, but not limited to, *Triticum timopheevii*, *Triticum monococcum*, *Triticum zhukovskyi* and *Triticum urartu* and hybrids thereof. Examples of *T. aestivum* subspecies included within the present invention are aestivum (common wheat), compactum (club wheat), macha (macha wheat), vavilovi (vavilovi wheat), spelta and sphaecrococcum (shot wheat). Examples of *T. turgidum* subspecies included within the present invention are turgidum, carthlicum, dicoccon, durum, paleocolchicuna, polonicum, turanicum and dicoccoides. Examples of *T. monococcum* subspecies included within the present invention are monococcum (einkorn) and aegilopoides.

[0192] In another embodiment, the present invention encompasses cytoplasmic and/or plastidic ACCases of barley plants or portions thereof. Two species of barley are most frequently cultivated, *Hordeum vulgare* and *Hordeum arizonicum.* Numerous other species are commercially important including, but not limited, *Hordeu bogdanii*, *Hordeum brachyantherum*, *Hordeum brevisubulatum*, *Hordeum bulbosum*, *Hordeum comosum*, *Hordeum depressum*, *Hordeum intercedens*, *Hordeum jubatum*, *Hordeum marinum*, *Hordeum marinum*, *Hordeum parodii*, *Hordeum pusillum*, *Hordeum secalinum*, *Hordeum spontaneum.*

[0193] In another embodiment, the present invention encompasses cytoplasmic and/or plastidic ACCases of rye plants or portions thereof. Two species of rye are most frequently cultivated, *Lolium canariense* and *Lolium edwardi*. Numerous other species are commercially important including, but not limited to, *Lolium multiflorum*, *Lolium perenne*, *Lolium persicum*, *Lolium remotum*, *Lolium rigidum*, and *Lolium temulentum*.

[0194] In another embodiment, the present invention encompasses cytoplasmic and/or plastidic ACCases of turfgrass or portions thereof. A turfgrass, as used herein, is a monocot, preferably a member of the Poaceae, that exhibits a growth habit as a ground-cover; such a growth habit is in many cases the result of a spreading or stoloniferous propagation pattern of the plant. Examples of genera to which turfgrass species and varieties belong inlcude: *Agropyron, Agrostis, Alopecurus, Ammophila, Andropogon, Arrhenatherum, Axonopus, Bouteloua, Bromus, Buchloe, Calamovilfa, Cenchrus, Chloris, Cynodon, Dactylis, Digitaria, Echinochloa, Ehrharta, Elymus, Eragrostis, Eremochloa, Festuca, Hilaria, Lolium, Muhlenbergia, Oryzopsis, Panicum, Paspalum, Pennisetum, Phalaris, Phleum, Poa, Setaria, Sorghastrum, Sorghum, Sporobolus, Stenotaphrum, Stipa, Trichachne, Tripsacum* and *Zoysia*. Numerous commercially important species of Turf grass include *Zoysia japonica*, *Agrostris palustris*, *Poa pratensis*, *Poa annua*, *Digitaria sanguinalis*, *Cyperus rotundus*, *Kyllinga brevifolia*, *Cyperus amuricus*, *Erigeron canadensis*, *Hydrocotyle sibthorpioides*, *Kummerowia striata*, *Euphorbia humifusa*, and *Viola arvensis*.

[0195] In yet other embodiments, the invention encompasses cytoplasmic and/or plastidic ACCases from monocot plants selected from *Zea* (for example *Zea mays*), *Sorghum* (for example *Sorghum bicolor*, *Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), pineapple (*Ananas comosus*), and banana species (*Musa* spp.) or portions thereof.

[0196] In other embodiments, the invention encompasses chimeric ACCases that have at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to a particular amino acid sequence for a monocot cytoplasmic and/or plastidic ACCase referenced herein.

[0197] In a specific embodiment, the invention encompasses a chimeric ACCase comprising an N-terminal region derived from a cytoplasmic ACCase from *Oryza sativa*.

[0198] In a specific embodiment, the invention encompasses a chimeric ACCase comprising a C-terminal region derived from a plastidic ACCase from *Oryza sativa*.

[0199] Methods of screening/growing/isolating yeast cells comprising a chimeric ACCase

[0200] Methods of culturing yeast cells are well known in the art. However, in some embodiments, the invention encompasses culturing yeast cells deficient in yeast *ACC1* while having a chimeric ACCase as described herein in an effort to induce, screen, grow, and/or isolate mutant ACCases comprising mutations that confer herbicide tolerance to the yeast cells.

[0201] In some embodiments, methods of the invention encompass culturing yeast cells in the presence of at least one herbicide in liquid or on solid media. In other embodiments, yeast cells are cultured in rich media prior to being exposed to at least one herbicide. In some embodiments, rich media may be supplemented with fatty acids, (including, but not limited to palmitic acid or steric acid) or biotin, alone or in combination. In some embodiments, yeast cells are cultured in media containing an increasing concentration of herbicide over time. In further embodiments, yeast cells are exposed to a stepwise increase of herbicide concentration over time.

[0202] In some embodiments, the methods of the invention encompass culturing yeast cells deficient in yeast *ACC1* while having a chimeric ACCase in the presence of at least one herbicide. In such embodiments, herbicides include, but are not limited to an aryloxyphenoxyproprionate (FOP) or cyclohexadione (DIM) herbicide. In some embodiments, FOP herbicides include, but are not limited to, of cyhalofop, quizalofop, diclofop, clodinafop, fluazifop, metamifop, propaquizafop, and fenoxyprop. In other embodiments, DIM herbicides include, but are not limited to, alloxydim, butroxydim, clethodim, cycloxydim, tepraloxydim, sethoxydim, tralkoxydim, and profoxydim. In yet other embodiments, the herbicide includes, but is not limited to, any herbicide listed in Table 1 herein.

[0203] In some embodiments, yeast cells are exposed to at least one herbicide present at a concentration from about 0.02 μM to about 200 μM. In other embodiments, yeast cells are exposed to at least one herbicide present at a concentration of about 0.02 μM, about 0.05 μM, about 0.1 μM, about 0.5 μM, about 0.1 μM, about 5 μM, about 10 μM, about 40 μM, about 100 μM, or about 200 μM. In specific embodiments, yeast cells are exposed to cycloxidim at a concentration of about 1μM, about 10 μM, about 40 μM, about 100 μM, or about 200 μM.

In another specific embodiment, yeast cells are exposed to tepraloxydim at a concentration of about 1μM, about 10 μM, about 40 μM, about 100 μM, or about 200 μM.

[0204] In some embodiments, yeast cells are exposed 1, 2, 3, 4, 5, 6, 7, 8, or more herbicides in one selection event. In other embodiments, yeast cells are exposed to at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, or more herbicides in one selection event. Typically, a selection event represents one round of selection conditions whereby a collection of cells are screened for, in this case, herbicide tolerance. In other embodiments comprising at least 2 or more herbicides, said herbicides may be present at a concentration that is not inhibitory to the chimeric ACCase when used in isolation from other herbicides. In yet other embodiments, 2 or more herbicides may be used concurrently or sequentially.

[0205] Methods of generating mutant chimeric ACCases

[0206] In some embodiments the invention encompasses mutant chimeric ACCases tolerant to at least one herbicide that arise due to mutations (for example, spontaneous or induced) of the chimeric ACCase present and selection in the presence of the at least one herbicide. In a specific embodiment, yeast cells are cultured in rich media prior to exposure to herbicide. In some embodiments, spontaneous mutagenesis may lead to at least one, at least two, at least three, at least four, at least five, at least six or more mutations that confer tolerance to the herbicide. In some embodiments, individual spontaneous mutations are sufficient to elicit tolerance to the herbicide used for selection. In other embodiments, mutations at multiple sites are required to provide tolerance to the herbicide tested. In yet other embodiments, the invention comprises using an ACCase with at least one known mutation that confers herbicide tolerance in a method to isolate or identify additional spontaneous mutations that increase or augment herbicide tolerance of the known mutation.

[0207] In a specific embodiment, the invention encompasses spontaneous mutation rates of about 1 tolerant mutation in every 5×10^9 cells.

[0208] In other embodiments, the invention encompasses a mutagenesis step to introduce mutations of the chimeric ACCase that will result in herbicide tolerance when yeast containing the mutated chimeric ACCase are cultured in the presence of the herbicide. In some embodiments, the mutagenesis step involves random mutagenesis of the nucleic acid encoding the chimeric ACCase.

[0209] In some embodiments, the chemical mutagenesis is performed by incubation of the yeast cell comprising the chimeric ACCase with a base analog mutagen. Base analog mutagens are chemicals that are structurally similar to normal bases and as such fool the DNA replication system. Their essential property is that they base-pair with two different bases thus making mutations because of their lack of consistency in base-pairing. To be mutagens they must be incorporated into the DNA and therefore they need be present during active DNA synthesis. One example is 5-bromo-deoxyuridine (5-BU), which can exist in two tautomeric forms: typically it exists in a keto form (T mimic) that pairs with A, but it can also exist in an enol form (C mimic) that pairs with G.

[0210] In other embodiments, the chemical mutagenesis is performed by incubation of the yeast cell comprising the chimeric ACCase with a base alkylating agent. Base alkylating agents react directly with certain bases and thus do not require active DNA synthesis in order to act but still do require DNA synthesis in order to be "fixed". They are very commonly used because they are powerful mutagens in nearly every biological system. Examples of alkylators include ethyl methane sulfonate (EMS), methyl methane sulfonate (MMS), diethylsulfate (DES), and nitrosoguanidine (NTG, NG, MNNG). These mutagens tend to prefer G-rich regions, reacting to form a variety of modified G residues, the result often being depurination. Some of these modified G residues have the property of inducing error-prone repair although mispairing of the altered base might also be possible. This stimulation of error-prone repair allows all sorts of mutation types to occur as a result of these mutagens, though base substitutions are by far the most frequent. It also appears that alkylated bases can mispair during replication.

[0211] In other embodiments, random mutagenesis is performed with UV radiation. In such methods, yeast cells comprising the chimeric ACCase sensitive to an herbicide is exposed to a UV source for a period of time sufficient to develop lesions in the DNA. Further protocols relating to UV induced mutagenesis can be found throughout the art including: Rose, M.D., Winston, F., and Hieter, P. 1990. Laboratory Course Manual for Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. which is hereby incorporated by reference for all purposes.

[0212] In other embodiments, the invention encompasses directed mutagenesis towards at least one or more amino acids. In some embodiments, directed mutagenesis may target 1, 2, 3, 4, 5, 6, 7, 8, or more amino acids in one event. In other embodiments, directed

mutagenesis may be to randomize the selection of an amino acid substitution for at least one or more amino acid present in the ACCase. In other embodiments, the directed mutagenesis is a restrictive substitution of at least one amino acid present in the ACCase. In other words, directed mutagenesis may be developed to include particular amino acids, or classes of amino acids while excluding others.

[0213] In one embodiment, the directed mutagenesis is performed by providing a library of oligonucleotides which hybridize to the nucleic acid encoding the chimeric ACCase, but are mutagenic for at least one amino acid position. The randomized library will hybridize to the chimeric ACCase gene and trigger recombination, thereby incorporating the randomization of at least one amino acid position in the chimeric ACCase gene. In some embodiments, the randomized library is a complete randomization for nucleotide base inclusion into the codon. In other embodiments, the randomized library is a restricted diversity library where the inclusion of nucleotides into the codon is not completely random, for example, the restriction of the first position being adenine with result in codons that code for amino acids that all begin with adenine. The resultant mutagenized chimeric ACCase gene can then be expressed and selected for herbicide tolerance as described herein.

[0214] Other suitable mutations that may be encoded in an oligonucleotide and used to to construct an ACCases to be used in the present invention include those in the following table.

Wild type amino acid and position	possible mutations
I1781	L, V, T, A
G1783	C
A1785	G
A1786	P
I1811	N
Q1824	P
A1837	V
V1864	F
W1999	C, G
W2027	C, R
E2039	G
I2041	N, V
V2049	F, I, L, C, T
A2059	V
W2074	L
V2075	M, L, I, GVV(double valine)
D2078	K, G, T
S2079	F
K2080	E, deleted

Wild type amino acid and position	possible mutations
I2081	deleted
C2088	R, W, F, G, H, K, S, T, L, V
K2095	E
G2096	A, S
V2098	A, G, P, H, S, C

[0215] In other embodiments, the invention encompasses mutant and chimeric ACCase wherein the at least one or more of the following specific substitutions is not present: I1781L, W2027C, I2041N, G2096A, D2078G and C2088R.

[0216] In a specific embodiment, the invention encompasses a yeast cell which comprises no active genomic ACCase gene, a nucleotide sequence encoding a chimeric ACCase as described herein, and at least one oligonucleotide that is mutagenic for at least one amino acid position in the chimeric ACCase.

[0217] In a specific embodiment, the invention encompasses compositions and methods where a chimeric ACCase is randomized at the amino acid corresponding to position 1781 (*Am*)of the plastidic ACCase of *Oryza sativa.* In other embodiments, the invention encompasses compositions and methods in which a chimeric ACCase is randomized at the amino acid corresponding to a position selected from 1781(*Am*), 1999(*Am*), 2049(*Am*), 2075(*Am*), 2075(*Am*), 2078(*Am*), 2098(*Am*), 2027(*Am*), 2041(*Am*), 2096(*Am*), and 2088(*Am*) of the plastidic ACCase of *Oryza sativa* (which are positions referenced to *Alopercurus myosuroides).*

[0218] Products

[0219] In some embodiments, the invention encompasses mutant ACCases which are tolerant to at least one herbicide. Typically, mutant ACCases are constructed providing an ACCase-deficient yeast with a chimeric ACCase. Generally, the chimeric ACCase comprising at least two regions, an N-terminal and a C-terminal region. In some embodiments the N-terminal region is derived from yeast, fungi or monocot cytoplasmic ACCases while the C-terminal region is derived from monocot plastidic ACCases and comprises an HSR. Once yeast cells complemented with the chimeric ACCase gene are cultured in the presence of herbicide, tolerant cells may be isolated. These tolerant cells may be studied to identify the mutation(s) not present in chimeric ACCase prior to culturing, which confers tolerance to at least one herbicide. Once the mutations conferring tolerance to

the chimeric ACCase are known, they may be introduced into a full-length monocot plastidic ACCase gene.

[0220] Methods for introducing the herbicide tolerant mutation in a full-length monocot plastidic ACCase gene include general molecular biology techniques known in the art. In some instances, the mutation can be excised from the chimeric ACCase gene via restriction endonucleases and 'spliced' into the full length monocot plastidic ACCase gene. Alternatively, the mutation can be engineered into the full length gene through PCR. Additionally, an oligo encoding the mutation may be generated and delivered to a host cell containing the full-length gene and, through homologous recombination, the mutation can be incorporated.

[0221] Full length monocot plastic ACCase genes containing the desired mutations can then be introduced into plants and/or plant cells using any method known to those skilled in the art. Typically the gene will included nucleotide sequences that direct expression of the gene in the desired plant cells and organelles. Such sequences include, but are not limited to, promoter sequences, leader sequences, targeting sequences (for example, chloroplast targeting sequences), terminator sequences and the like. The genes may be introduced using any methods known to those skilled in the art including, but not limited to, by using tumor-inducing (Ti) plasmid vectors, plant transformation vectors, PEG mediated protoplast transformation, electroporation, microinjections, and biolistics or microprojectile bombardment. Plant cells comprising the mutated ACCase gene may then be regenerated into plants using known techniques. The regenerated plants will comprise the mutated ACCase gene. Typically, the mutated gene will render the plant herbicide resistant. In some embodiments, the ACCase gene comprising one or more substitutions will be introduced into the wild type locus of the ACCase gene.

[0222] Methods for determining herbicide sensitivity

[0223] In some embodiments of the invention, chimeric ACCases invention comprise an HSR derived from a monocot plastidic ACCase, rendering the chimeric ACCase herbicide sensitive. To determine if a monocot plastidic ACCase is suitable for use in constructing an herbicide-sensitive chimeric ACCase, the sensitivity of the monocot plastidic ACCase to inhibition by an ACCase-inhibiting herbicide may be determined using any assay method known to those skilled in the art.

[0224] The assay can be, e.g., either (1) a whole-plant assay, e.g., a visual injury rating scale, scored following contact with a 1x rate of ACCase-inhibitor selected from among the DIMs, FOPs, and DENs, according to manufacturer's instructions; or (2) a cell-based assay (e.g., plant cell-based or microbial cell-based) wherein the cells are exposed to equivalent concentrations of ACCase inhibitor as are delivered to in planta cells in the plant-based assay, taking into account the increase in delivery of herbicide active ingredient(s) to the cells of an in vitro cell-based assay. In either the whole-plant or cell-based assay, the ACCase whose herbicide susceptibility is being determined can be native to the plant or cell or can be exogenous thereto. The degree of herbicide-induced injury to the plant or cell/cell culture provides an indication of whether or not the monocot plastidic ACCase therein is to be considered "susceptible" to the herbicide.

[0225] WHOLE PLANT (PVHI) ASSAY EXAMPLE

[0226] For example, in a whole-plant assay to determine herbicide susceptibility, a visual injury rating scale of 0 to 10 can be used, wherein 0 indicates no visually detectable injury symptoms, and 10 indicates death of the plant. Use of such a scale to score plant "injury" can take into account symptoms including, e.g., chlorosis or other discoloration, necrosis, wilting, stunting and other plant deformity. In one exemplary 0-to 10 plant injury rating, plants rated from 0 to 3 = tolerant, with 0 to 1 = highly tolerant; plants rated 4 to 10 = susceptible, with 8 to 10 = highly susceptible.

[0227] In an exemplary embodiment, an herbicide sprayer calibrated to deliver a spray volume of typically about 100-500 L/ha (usually about 250 L/ha) can be used and, following treatment, the injury determination can be made at least once within 4 weeks after treatment. An example of such a visual injury rating scale is as follows:

0	no visible symptoms
1	slight symptoms
2	minor symptoms
3	mild symptoms, with agronomically-acceptable appearance maintained
4	evident symptoms, with no reduction in biomass
5	obvious symptoms, with likely reduction in biomass
6	substantial symptoms, with definite reduction in biomass
7	25-50% of plant tissue exhibits necrosis
8	50-75% of plant tissue exhibits necrosis
9	>75% of plant tissue exhibits necrosis
10	total plant death

[0228] Such an assay can be referred to herein as a Plant Visual Herbicide Injury (PVHI) assay.

[0229] CELL-BASED (CBHI) ASSAY EXAMPLE

[0230] In the case of cell-based assay to determine herbicide susceptibility, a post-herbicide-treatment rating scale can be used that ranges, e.g., from 0 to 6, with 0 indicating no symptoms and 6 indicating death of all cells, wherein a score of 0-2 indicates tolerant, with 0-1 being highly tolerant; and 3-6 indicating susceptible, with 5-6 being highly susceptible. In scoring "injury" for cells, symptoms to be noted include, e.g., temporary decrease in rate of cell multiplication, decrease in production of normal metabolites (e.g., fatty acids, phospholipids), increase in production of stress-related metabolites (e.g., abscisic acid, ethylene, GABA), sustained decrease in rate of cell multiplication, decrease in plant cell chloroplast population, plant cell senescence, chloroplast localization in the plant cell vacuole, cell membrane deformation or disruption, cell leakage, and cell lysis. In an exemplary embodiment, the scoring can be performed at least once within 10 days following exposure to the herbicide. In the case of such a cell-based assay, the visual scoring can be done by eye alone, or with the aid of a microscope, metabolic screen, dye or contrast agent, or other analytical equipment or supplemental agent.

[0231] Such an assay can be referred to herein as a Cell-Based Herbicide Injury (CBHI) assay.

6. EXAMPLES

[0232] The invention is now described with reference to the following examples. These examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these examples but rather should be construed to encompass any and all variations which become evident as a result of the teachings provided herein.

[0233] Example 1. Construction of Yeast ACCase Screening Cell Line

[0234] Summary: For the invention described here, we constructed a rice (*Oryza sativa*) chimeric ACCase gene (OsJACCc60p40) corresponding to the N-terminal 60% of the rice cytoplasmic ACCase gene fused to the C-terminal 40% of the rice plastidic ACCase gene. A low copy number CEN plasmid with OsJACCc60p40 (where c60 = cytoplasmic 60% and p40 = plastidic 40%) was introduced into the yeast heterozygous diploid YNR016C BY4743 (acc1::kanMX/ACC1), which was equipped with the SGA reporter for plasmid-chromosome

shuffling (Tong et al., 2001). After sporulation and germination on selective medium, MATa haploid cells relying solely on rice OsJACCc60p40 for fatty acid biosynthesis were obtained. These cells can be used to isolate mutations by plating cells directly on medium containing herbicide. These cells were made competent for LiAc/PEG-based transformation (Gietz et al., 1992) of DNA fragment mixtures that could create mutant chimeric ACCase genes through homologous recombination. We demonstrated that directed and spontaneous mutagenesis leads to herbicide tolerant chimeric ACCases.

[0235] Yeast strains and growth conditions

[0236] The heterozygous diploid YNR016C BY4743 (MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS met15Δ0/MET15 ura3Δ0/ura3Δ0 can1Δ::LEU2-MFApr-HIS3/CAN1 acc1::kanMX/ACC1 [Chromosome: 14]) was ordered from Open Biosystems (catalogue number YSC4034-97040689, clone ID 25391). YNR016C BY4743 was grown in YPG (20 g/L yeast extract, 40 g/L bacto tryptone, 2% filter-sterilized galactose, pH 6.5) with 200 mg/l G418 (Invitrogen, Carlsbad, CA) at 30°C and 200 rpm. Haploid strains, which depend on plasmid-borne plant ACCase for fatty acid biosynthesis, were grown in 1 g/L monosodium L-glutamic acid, 1.7 g/L yeast nitrogen base without amino acids and without ammonium sulfate (BD, Franklin Lakes, NJ), 1.57 g/L SC-Arg-His-Leu-Ura (Sunrise Science, San Diego, CA), 2% galactose, 200 mg/L G418 and 50 mg/L L-Canavanine (Sigma-Aldrich, St. Louis, MO) at 30°C and 200 rpm. The control haploid strain which depends on yeast *ACC1* for fatty acid biosynthesis was grown in 1 g/L mono sodium L-glutamic acid, 1.7 g/L yeast nitrogen base without amino acids and without ammonium sulfate, 1.57 g/L SC-Arg-His-Leu-Ura, 2% galactose, 50 mg/L L-Canavanine and 0.02 g/L Ura at 30°C and 200 rpm. For growth on plates, all media were supplemented with 20 g/L Difco Agar Noble 214230. For all media, with the exception of YP, agar was autoclaved separate from the other solutions as suggested by Tong and Boone (2006). Cycloxydim was kept as a 614 mM stock solution in naphtha or a 100 mM stock solution dissolved in methanol. Both haloxyfop and tepraloxydim were kept as 100 mM solutions in DMSO.

[0237] Plasmid construction

[0238] A fusion construct of the GAL10 promoter, OsJACCc60p40 and the ADH1 terminator (Figure 1B and 2C) was synthesized by GENEART (Regensburg, Germany). The nucleotide sequence of OsJACCc60p40 was optimized for expression in yeast with Leto 3.0 software (Entelechon, Regensburg, Germany). Restriction sites for mutagenesis and

subcloning were introduced at key points (Figure 1B) and homology with yeast *ACC1* was kept to a minimum to avoid gene conversion. The construct was delivered as purified plasmid in the pMK vector (RTP3240, Figure 2A). The yeast expression vector pRS416 (Mumberg et al., 1995) was modified prior to cloning of the 7468 bp SalI-SpeI fragment of RTP3240 containing GAL10pr-OsJACCc60p40-tADH1. The NotI and Acc65I site in the multiple cloning site of pRS416 were sequentially eliminated by digestion with the corresponding enzymes, 'blunting' with T4 DNA polymerase (Promega, Madison, WI) and re-ligation. The adapted pRS416ΔNotIΔAcc65I plasmid was digested with XhoI and SpeI and RTP4108 was constructed by ligation of the 7468 bp SalI-SpeI fragment of RTP3240 (Figure 2B and 2C).

[0239] The complete rice plastidic ACCase coding sequence was also synthesized by GENEART and delivered as RTP3378 (Figure 3A). This ACCase sequence (Figure 3B) was optimized for expression in rice and most of the restriction sites present in RTP4108 were included in corresponding positions to allow exchange of subfragments. The triplet at relative position 1781 in RTP3378 was synthesized to encode leucine rather than the 'wild type' amino acid isoleucine (I1781L). A 134 bp Acc65I-XhoI fragment of RTP3378 with the I1781L mutation was exchanged with the corresponding fragment in RTP4108 to produce the control plasmid RLW001. C-terminal 10x His tags were added to both RLW001 and RTP4108 for plasmids RTP4106 and RTP4107, respectively by exchanging the BamHI-NotI fragments at the 3' coding end with the BamHI NotI-digested synthetic sequence:

[0240] SEQ ID NO:1

ggatccaactttgattgacttgaaggctaagttggaagttgccaacaagaatggatctgctgatacgaagtctttgcaagaaaatattgaa
gctagaactaagcaactgatgccattatacacccaaattgctatcagattcgctgaattgcatgatacctctttgagaatggctgctaagg
gtgttatcaagaaggttgttgattgggaagaatccagatctttcttctacaagagattgagaaggagaatttccgaagatgttttggctaag
gaaattagagctgttgctggtgaacaattctctcatcaaccagctattgaactgattaagaagtggtactctgcttctcatgctgcagaatg
ggatgatgatgatgctttcgttgcttggatggataacccagaaaactacaaggactacattcaatacctgaaagctcaaagagtgtctca
atctttgtcctctttgtctgattcctcttctgatctacaagctctaccacaaggtttgtctatgttgttggataagatggacccatctagaagag
ctcaattggttgaagaaatcagaaaggttttgggtcatcatcatcatcatcatcatcatcattgataagcggccgc (RTP3889,
10x His codons underlined, Figure 4).

[0241] Transformation of plasmids harboring rice chimeric ACCase gene constructs

[0242] Transformation was carried out with the Yeastmaker™ Yeast Transformation System 2 kit (Clontech, Mountain View, CA) as described in Clontech's manual with 200 ng plasmid. Cells were plated on 20g/l Difco Agar Noble 214230, 1 g/L monosodium L-

glutamic acid, 1.7 g/L yeast nitrogen base without amino acids and without ammonium sulfate, 1.57 g/L SC-Arg-His-Leu-Ura, 2% galactose, 200 mg/L G418 and 0.02 g/L His.

[0243] Sporulation and plasmid-chromosome shuffling

[0244] Single colonies of the heterozygous diploid YNR016C BY4743 harboring a plasmid with rice chimeric ACCase were grown in 10 mL 1 g/L monosodium L-glutamic acid, 1.7 g/L yeast nitrogen base without amino acids and without ammonium sulfate, 1.57 g/L SC-Arg-His-Leu-Ura, 2% galactose, 200 mg/L G418 and 0.02 g/L His at 30°C and 200 rpm until the OD600 nm reached 0.400. Cell cultures were transferred to 50 mL tubes, centrifuged (700x g, 5 minutes) and washed in 40 ml H_2O. The wash step was repeated and cells were resuspended in 2 ml sporulation medium (1% KAc, 0.005% ZnAc, 0.3 mM His). Tubes were shaken horizontally at 25°C, 200 rpm for 5 days. Cells were collected through centrifugation, washed with 40 ml H_2O and resuspended in 10 mL H_2O after the final centrifugation step. 50 µL and 50 µL of 10x and 100x dilutions were plated on 20 g/L Difco Agar Noble 214230, 1 g/L monosodium L-glutamic acid, 1.7 g/L yeast nitrogen base without amino acids and without ammonium sulfate, 1.57 g/L SC-Arg-His-Leu-Ura, 2% galactose, 200 mg/L G418 and 50 mg/L L-Canavanine and incubated at 30°C for 5 days. To obtain the control haploid strain which depends on yeast *ACC1* for fatty acid biosynthesis the same procedure was used with the following changes: Initial growth took place in YPG with 200 mg/mL G418 and plates for selection of haploids did not contain G418, but were supplemented with 0.02 g/L Ura.

[0245] Confirmation of genotype

[0246] Cells from 1 mL of culture were washed twice with 1 mL TE buffer. Following the last centrifugation step, excess liquid was aspirated and the cells were resuspended and disrupted by freeze-thawing. Subsequently, samples were heated at 95°C for 5 min with occasional shaking. Cell debris was removed by centrifugation for 5 min and 2 µL of the denatured genomic DNA sample was used to confirm the genotype by PCR amplification with rice chimeric ACCase-specific primers (c60p40-5924FW: ttctcacgtcaagattccacc [SEQ ID NO:3] and c60p40-6421RV: tactcaccagtttccatagag [SEQ ID NO:4]) and ACC1-specific primers (ACC1aFW: gtgttgacaccgttcacgtgg [SEQ ID NO:5]and ACC1aRV: caccggagaccatttccgttg [SEQ ID NO:6]; ACC1bFW: gtttggctccagaagtacatc [SEQ ID NO:7] and ACC1bRV: tcatggtcgcttctgatcttt [SEQ ID NO:8]). PCR was performed with the Expand

High Fidelity PCR system (Roche, Mannheim, Germany) according to the manufacturer's instructions.

[0247] Mutagenesis with DNA fragments containing degenerate nucleotides

[0248] Fragment mixtures with degenerate nucleotides in selected positions were ordered from Epoch Biolabs (Sugar Land, TX). A fragment designated LN1-mut spanned 1169 bp of the 3' coding end of OsJACCc60p40 and had the following composition:

[0249] SEQ ID NO:2

tacctctgttattgctcacaagatgcaacttgattctggtgaaattagatgggtcattgattccgttgttggtaaggaagatggtttgggagtt gaaaatnnncatggttctgctgcaattgcttctgcttactcgagagcttacaaggaaacgttcactttgactttcgttactggtagaactgtt ggtattggtgcttacttggctagattgggtatcagatgcatccaaagacttgatcagcctattatcttgactggttactctgctttgaataagtt gttgggtagagaagtttactcgtctcatatgcaattgggtggaccaaagattatggcaacaaatggtgttgtacacttgactgtttctgatg acttggaaggtgtctctaatatcctgagatggttgtcttacgttccagcttacattggtggtcctttgccagttactactccattggacccacc tgatagaccagttgcttacatacctgaaaactcttgcgatccaagagctgcaattagaggtgttgatgactctcaaggtaagtggcttggt ggcatgttcgataaggattccttcgttgaaactttcgaaggttgggctaagactgttgttactggtagagctaagctaggaggtattccagt tggtgttattgcagttgaaacgcaaactatgatgcaaactattccagctgacccaggtcaattggattctagagaacaaagtgttcctaga gctggtcaagttnnnnttcccagattctgctacaaagactgctcaagctttgttggacttcaatcgcgaaggtttgccattgttcatcttggca aatnnnagaggtttctccggaggtcaaagagatttgttcgaaggtnnnttgcaagctggttctactatcgtcgaaaacttgagaacctac aatcaaccagctttcgtttacattcctatggctgctgaattgagaggtggcgcctgggttgttgttnnntctaagattaacccggaccgtat cgaannntacgctgaacgtacggctaagggtaatgttttggaaccacaannnttgattgaaatcaagttcagaagtgaagaattgcaa gattgcatgagtagattggatccaactttgattgacttgaaggctaagttggaagttgccaaca

[0250] In one study only degeneracy in the triplet that corresponds to isoleucine at relative position 1781 was tested (nucleotides underlined). A 299 bp fragment mixture was amplified with the Expand High Fidelity PCR system using LN1-mut as template and c60p40-10451FW (tacctctgttattgctcacaa [SEQ ID NO:9]) and c60p40-10750RV (taaacttctctacccaacaac [SEQ ID NO:10]) as primers.

[0251] In other experiments, each degenerate triplet was flanked by 150 bp OsJACCc60p40 DNA to allow incorporation through homologous recombination except the triplet corresponding to I1781. PCR was performed with the Expand High Fidelity PCR system using primers that match the outer 21 nucleotides of the 303 bp synthetic fragments. For I1781, the degenerate triplet was flanked by 100 bp at the 5' coding end and 197 bp at the 3' coding end in the final PCR product.

[0252] Preferably, the mutated triplet is centered in the synthetic fragment. In our first experiments with substitutions for I1781, the first set of synthetic fragment mixtures that was ordered contained large fragments with multiple mutations at multiple positions. That was used as template for the I1781 fragments in a PCR reaction (excluding the other mutations that were present). Later, synthetic, symmetric fragments for each position were used.

[0253] After PCR, primers and dNTPs were removed with the Wizard® SV Gel and PCR Clean-Up System (Promega) and the PCR product was checked on gel. Approximately 200 ng was used for transformation. When mixtures of oligonucleotides are being transformed, the scale of the transformation procedure may be increased to ensure each of the components in the mixture will be represented among the transformed colonies. For mixtures we used 6 x 5 µg in parallel using the Yeastmaker® library scale protocol (per herbicide). When a single mutation is introduced (for example I1781L) by using a purified single fragment, only one transformed colony is required on a plate. Thus the scale can be much lower, e.g 200 ng and the standard transformation protocol may be used.

[0254] Cells that rely on RTP4108 for fatty acid biosynthesis (designated RTP4108-8b-2) were pre-grown in YPG G418 or YPG G418 supplemented with 1% Tween, 0.015% palmitic acid, 0.015% stearic acid and/or 0.4 mg/L biotin. Prior to transformation cells were washed 2x with H_2O to remove fatty acids from the medium. Transformation was performed as described in the Yeastmaker™ Yeast Transformation System 2 kit protocol with a 1½ hour recovery step in YPG. For each fragment mixture 6 transformations were plated onto medium with tepraloxydim and 6 onto medium with cycloxydim. Thus, 12 transformations at Yeastmaker's library scale are started simultaneously for each fragment mixture and they are treated the same until it is time to plate them on their respective herbicide plates. Control DNA consisted of the same fragment in which *nnn* was ATT (encoding isoleucine, therefore creating the original sequence present in RTP4108) and TTG (encoding leucine, therefore creating a positive control for transformation).

[0255] DNA isolation, PCR of the 3' coding end and sequencing of candidate herbicide tolerant genes

[0256] Single colonies growing on plates with herbicide were transferred to fresh plates and selected once more on medium with the same herbicide to remove any traces of unwanted, untransformed cells. Cells were subsequently grown in 1 mL liquid medium (1 g/L mono sodium L-glutamic acid, 1.7 g/L yeast nitrogen base without amino acids and without

ammonium sulfate, 1.57 g/L SC-Arg-His-Leu-Ura, 2% galactose, 200 mg/L G418 and 50 mg/L L-Canavanine) without herbicide until the $OD_{600\ nm}$ reached 0.400. DNA was isolated with Zymoprep™ II, Yeast Plasmid Miniprep Kit (Zymo Research, Orange, CA). PCR was performed with the Expand High Fidelity PCR system and c60p40-9561FW (ccgttctctagaactattgac [SEQ ID NO:11]) and c60p40-12331RV (cgacctcatgctatacctgag [SEQ ID NO:12]) primers. Sequencing of PCR products was performed by DNA Landmarks. For high throughput sequencing cultures were grown in 1 ml medium in 96 deepwell blocks mounted on the shaker for 2 days. 0.4 ml of the fully grown yeast cultures was spun down and the pellets were resuspended in 30 ul zymolyase solution (2.5 mg/ml ICN zymolyase, 1.2 M Sorbitol, 0.1 M Na Phosphate pH 7.4). 10 ul of that suspension was aliquoted in V-shaped bottom 96-well plates and frozen on dry ice before shipping to DNA Landmarks. A stretch of DNA ranging from 227 bp upstream from the codon for I1781 to 194 bp downstream form the codon for V2098 was PCR-amplified (primers CTGGTGCAAGAATCGGTATT and TGGGTGTATAATGGCATCAGT) and sequenced.

[0257] High throughput production of growth curves in the absence and presence of herbicides

[0258] Single colonies of mutants and controls were grown in 1 g/l mono sodium L-glutamic acid, 1.7 g/l yeast nitrogen base without amino acids and without ammonium sulfate, 1.57 g/l SC-Arg-His-Leu-Ura, 2% galactose, 50 mg/l L-Canavanine and 200 mg/l G418 (rice hybrid ACCase-dependent strains) or 0.02 g/l Ura (ACC1-dependent strain) at 30°C and 200 rpm for two days. Fully grown cultures were spun down and resuspended in 300-800 µl medium to produce starting strains with roughly comparable densities. 5 µl of starting strain was transferred in triplicate to 96 deepwell blocks with 1.5 ml of the same medium or the same medium supplemented with herbicide. Blocks were mounted on the shaker and 100 µl samples were taken every 24 hours to determine the optical density at 600 nm with the Multiskan Ascent (ThermoLabs) plate reader.

[0259] Results and discussion

[0260] Design of OsJACCc60p40 and construction of RTP4108

[0261] The sequences of the rice (*Oryza sativa* Japonica group) plastidic and cytoplasmic ACCase genes (Accession numbers EAZ33685 and AAM18728, respectively) were used to design the synthetic, chimeric ACCase gene OsJACCc60p40 (Figure 1B and 2C). The protein encoded by OsJACCc60p40 consists of the first 60% of the cytoplasmic ACCase protein and

the last 40% of the plastidic ACCase protein. The sequence of the plastidic ACCase protein that was submitted to GenBank (EAZ33685) contained sequence errors, which were corrected with the help of ref|NC_008398.1|. With these corrections the amino acids sequence was found to be identical to that of the plastidic ACCase protein of the Indica group (EAY97401). The GAL10pr-OsJACCc60p40-tADH1 fusion construct was synthesized and cloned into a modified pRS416 shuttle vector (Mumberg et al., 1995) to create RTP4108. RTP4108 and all other plasmids that contain the GAL10pr-OsJACCc60p40 fusion construct or derivatives thereof are highly toxic to *E. coli* and prolonged growth of bacterial cultures results in gross rearrangements within the plasmid. Therefore, identical *E. coli* cultures were grown in parallel in five culture tubes in the presence of glucose until early log phase after which samples were checked for rearrangements. All culture tubes in which rearrangements were not apparent on gel after HindIII digestion were pooled and subjected to plasmid isolation. Maintenance of plasmids in *E. coli* was minimized to this step only. Next to RTP4108 (GAL10pr-OsJACCc60p40), we also constructed RTP4107 (GAL10pr-OsJACCc60p40 + 10x C-terminal His tag), RLW001 (GAL10pr-OsJACCc60p40 I1781L) and RTP4106 (GAL10pr-OsJACCc60p40 I1781L + 10x C-terminal His tag)(Figure 4).

[0262] Transformation of the heterozygous diploid YNR016C BY4743 (acc1::kanMX/*ACC1*), plasmid-chromosome shuffling and complementation of *ACC1* deficiency by OsJACCc60p40

[0263] Yeast mutants in which the endogenous *ACC1* gene is deleted are not viable. We used a plasmid-chromosome shuffling method to replace the *ACC1* gene with plasmid-borne rice chimeric ACCase (Figure 5a-5e). The low copy number plasmids RTP4108, RTP4107, RLW001 and RTP4106 were transformed to the heterozygous diploid YNR016C BY4743 and cells were selected for the presence of the URA3 gene on the plasmid (Figure 5b-5d). Thus, the diploid cells contained both the endogenous *ACC1* gene and a rice chimeric ACCase. Shuffling was made possible due to two heterozygous loci: (i) on one of the chromosomes 14, the endogenous *ACC1* gene had been replaced by the kanMX cassette, which confers resistance to G418 and (ii) on one of the chromosomes 5 the CAN1 gene had been replaced by the SGA reporter (can1Δ::LEU2-MFA1pr-HIS3/CAN1, Tong et al., 2001). Sporulation was induced in diploid cells which contained both ACCase genes. After sporulation cells were plated on selective medium and grown at 23°C and 30°C (Figure 5d-5e). Haploid cells which inherited the endogenous *ACC1* gene were killed by G418. All diploid cells still present in the sporulation mix and all haploid cells which possessed the

CAN1 gene were killed by L-Canavanine; a toxic arginine analogue that is imported into the cell by the CAN1 gene product. The SGA reporter consists of the auxotrophic HIS3 selection gene under control of the MATa-specific MFA1 promoter. The absence of histidine in the medium ensured that only MATa haploid cells were able to survive. Haploid cells with RTP4108, RTP4107, RLW001 and RTP4106 were checked for the presence of rice chimeric ACCase and the absence of *ACC1* with primer sets specific for both genes. The genotype of multiple independent colonies for each of these haploid cells was confirmed (data shown for RTP4108 in Figure 6). All four plasmids complemented the acc1 knockout at both 23°C and 30°C. The wheat ACCc60p40 construct used by Nikolskaya et al. (1999) was not able to complement at 30°C, despite being expressed from a high copy number plasmid. The result obtained here underlined the solid design of the rice chimeric ACCase nucleotide sequence and expression system, although the observed difference in growth at 30°C could also be caused by specific differences in amino acid sequence between the chimeric ACCases from rice and wheat. Sporulation was also induced with diploid cells that did not contain a plasmid to obtain a haploid strain that relied on *ACC1* (designated yeast *ACC1*, Figure 6).

[0264] 'Kill curves' for yeast strains containing various ACCase variants with cycloxydim and tepraloxydim

[0265] Model strains with RTP4108 (RTP4108-8b-2), RTP4107 (RTP4107-5b-1), RLW001 (also referred to as RLW001-1a-1, or YIL-1a-1) and RTP4106 (RTP4106-7a-1) and yeast *ACC1* were plated on different concentrations of haloxyfop, cycloxydim and tepraloxydim (Figure 7) and incubated at 30°C for 4 days. Growth of strains with I1781 was severely hampered by 100 µM cycloxydim and 1 µM tepraloxydim. The mutation I1781L, which has been known to confer tolerance in plants, increased the tolerance and significant reduction in growth was observed at 200 µM cycloxydim and 10 µM tepraloxydim. This test established that yeast could be used as a model organism to screen for increased cycloxydim and tepraloxydim tolerance. As expected, yeast *ACC1* was tolerant to all concentrations tested, although a slight, yet reproducible reduction in growth was observed at 200 µM cycloxydim. Control plates with comparable volumes of DMSO and methanol showed no effect on the growth of any of the strains used here (data not shown). His-tags did not have a detrimental effect. Hence, mutagenesis can also be done starting with RTP4107-5b-1 or RTP4106-7a-1 (the latter for finding double mutations with I1781L) and the presence of the His-tags could be beneficial for subsequent biochemical characterization. The results shown

in Figure 7 were highly reproducible with 3-4 independent haploid lines which contained the same plasmids (data not shown).

[0266] Mutagenesis with fragments containing degeneracy at preselected sites

[0267] RTP4108-8b-2 was chosen for a pilot transformation experiment with a 299 bp fragment corresponding with the sequence of RTP4108 with TTG, encoding I1781L, in the center (Mu13). A similar, 299 bp control fragment contained the original ATA codon for isoleucine at that position (Wt10). RTP4108-8b-2 cells were initially grown in monosodium L-glutamic acid, yeast nitrogen base without amino acids and without ammonium sulfate, SC-Arg-His-Leu-Ura, 2% galactose, 200 mg/l G418 and 50 mg/l L-Canavanine, but the transformation efficiency turned out to be impractically low due to the slow growth rate in this medium (data not shown) and possibly altered membrane composition (Schneiter et al., 1996). Table 2 shows the transformation efficiency after pregrowth in rich medium or rich medium supplemented with FA and/or biotin and plating on medium with 100 μM cycloxydim. Pregrowth in YPG G418 + FA and biotin resulted in 11 transformants and this number can be increased to 200-300 colonies by switching from the '200 ng' to the 'library scale' (15 μg) transformation protocol described in Clontech's Yeastmaker™ Yeast Transformation System 2 kit manual (data not shown). Transformation with a 299 bp fragment mixture containing all possible triplets at the site corresponding to I1781 has been carried out.

[0268] Table of transformation efficiencies after pre-growth in different media

Fragment transformed	Pregrowth	Plating on	# colonies
Wt10	YPGal G418	100 μM cycloxydim	0
Wt10	YPGal G418 Biotin	100 μM cycloxydim	0
Wt10	YPGal G418 FA	100 μM cycloxydim	2
Wt10	YPGal G418 Biotin FA	100 μM cycloxydim	0
Mu13 (I1781L)	YPGal G418	100 μM cycloxydim	5
Mu13 (I1781L)	YPGal G418 Biotin	100 μM cycloxydim	7
Mu13 (I1781L)	YPGal G418 FA	100 μM cycloxydim	6
Mu13 (I1781L)	YPGal G418 Biotin FA	100 μM cycloxydim	11

[0269] A series of such experiments were performed. In pilot experiments the efficiency was typically only 5-20 transformants/μg DNA (data not shown). The haploid strains RTP4108-8b-2 and RTP4107-5b-1 (as well as RLW001-1a-1 and RTP4106-7a-1) have a doubling time of approximately 4½ hours in liquid YPG G418 biotin FA medium. The slow

growth rate and possibly altered membrane composition (Schneiter et al., 1996) are believed to be the primary reasons for the poor transformation efficiencies. The transformation efficiency was ~20% higher when pre-growth of cells took place in YPG medium supplemented with biotin and fatty acids and recovery took place in YPG medium as opposed to Clontech's standard YPD plus transformation recovery medium. Despite the improvements, transformation rates were poor, thus hampering efficient screening of high complexity mutagenized fragment mixtures.

[0270] Spontaneous mutants and sequence analysis

[0271] The table of transformation efficiencies above showed that transformation with Wt10 resulted in the growth of two colonies when cells were pregrown in YPG G418 + fatty acids. In theory, the Wt10 PCR-amplified fragment could contain unintentional mutations, either introduced by Epoch Biolabs during synthesis or by PCR with Expand DNA polymerase, which could incorporate into OsJACCc60p40 through homologous recombination and give rise to herbicide tolerant colonies. However, it was found that direct plating of RTP4108-8b-2 on 200 μM cycloxydim or 10 μM tepraloxydim, resulted in 1 spontaneous mutant for every ~5x 10^9 cells. This phenomenon was fully exploited and RTP4108-8b-2 was plated in large quantities on 200 μM cycloxydim and 10 μM tepraloxydim. Similarly, RLW001-1a-1 was plated on 40 μM tepraloxydim and 100 μM tepraloxydim to obtain double mutants. In addition, all colonies that grew on control plates in earlier experiments aimed at optimizing the transformation procedure were analyzed (data not shown). All herbicide-tolerant colonies were grown on plates with 100 and 200 μM cycloxydim and 1 and 10 μM tepraloxydim and the relative growth rate was estimated. The following table summarizes other colonies of interest for which sequence data was obtained.

[0272] Table of mutants obtained in various screens (left column) and tolerance to C100 (100 μM cycloxydim), C200 (200 μM cycloxydim), T1 (1 μM tepraloxydim) and T10 (10 μM tepraloxydim).

			Relative growth on medium after transfer					
Identifier	Starting haploid cells	Isolated from plate with	No herb.	C100	C200	T1	T10	Substitution
RTP4108-8b-2 control	RTP4108-8b-2	No herbicide	++++	-	-	-	-	None
RLW001-1a-1 control	RLW001-1a-1	No herbicide	++++	+++	++	++	+	I1781L
8	RTP4108-8b-2	100 μM cycloxydim	++++	++	++	+++	+	W1999G
22	RTP4108-8b-2	100 μM cycloxydim	++++	+++	++	-	-	I1781T
29	RTP4108-8b-2	1 μM tepraloxydim	++++	+	-	++	+	V2049F
30	RTP4108-8b-2	1 μM tepraloxydim	++++	+	-	+++	+	V2049F
32	RTP4108-8b-2	1 μM tepraloxydim	++++	+	-	+++	+	V2049F
33	RTP4108-8b-2	1 μM tepraloxydim	++++	+	+	+++	+	V2049F
18	RTP4108-8b-2	10 μM tepraloxydim	++++	+	-	++++	+++	V2075L
24	RTP4108-8b-2	1 μM tepraloxydim	++++	++	+	+++	+++	V2075L
25	RTP4108-8b-2	1 μM tepraloxydim	++++	++	+	+++	+++	V2075L
26	RTP4108-8b-2	1 μM tepraloxydim	++++	++	+	+++	+++	V2075L
27	RTP4108-8b-2	1 μM tepraloxydim	++++	++	-	+++	+++	V2075L
57	RTP4108-8b-2	10 μM tepraloxydim	++++	++	+	+++	+++	V2075L
58	RTP4108-8b-2	10 μM tepraloxydim	++++	++	+	+++	+++	V2075L
34	RTP4108-8b-2	1 μM tepraloxydim	++++	++	+	++	+	V2075I
31	RTP4108-8b-2	100 μM cycloxydim	++++	+++	+++	+++	+++	D2078G
21	RTP4108-8b-2	10 μM tepraloxydim	++++	+++	+++	+++	+++	V2098A
28	RTP4108-8b-2	200 μM cycloxydim	++++	+++	++	+++	++	V2098A
78	RLW001-1a-1	40 μM tepraloxydim	++++	++++	++++	++++	++++	I1781L + V2049F

[0273] Mutant 8 possessed a new mutation, W1999G, which gave tolerance to both cycloxydim and tepraloxydim. The mutation W1999C has been associated with fenoxaprop tolerance, but this mutation did not lead to tolerance for sethoxydim and tralkoxydim (Liu et al., 2007).

[0274] I1781T in mutant 22 also represented a new mutation at a known position. Interestingly, this mutation only conferred tolerance to cycloxydim. V2049F led to moderate tolerance to tepraloxydim. This single mutation was found in four independent colonies (29, 30, 32 and 33) and the growth rates on plates with different herbicides was highly reproducible.

[0275] Likewise, the mutation V2075L, which led to high tepraloxydim tolerance only, was found multiple times and led to consistent growth rates as shown above. The cycloxydim

tolerance-conferring mutation I1781T and tepraloxydim tolerance-conferring mutation V2075L may be used in rotation in rice.

[0276] The mutation V2075I was less favorable for DIM tolerance compared to V2075L.

[0277] Mutant 31 contained a known mutation: D2078G. This mutation has been known to be associated with a fitness penalty in rice. In yeast, the fitness penalty does not translate itself in a slow growth rate (Liu et al., 2007 and this study). However, reducing GAL10 promoter activity by decreasing the galactose concentration in the medium may reveal suboptimal enzymatic activity of ACCase with D2078G.

[0278] V2098A was indentified twice and led to high tolerance to both cycloxydim and tepraloxydim.

[0279] In one experiment, in which RLW001-1a-1 was plated on 40 µM tepraloxydim, a tolerant double mutant with I1781L was uncovered. The second mutation turned out to be V2049F. The double mutant performed better than each of the two single mutants separately on medium with DIMs.

[0280] The isolation of ACCases with the same mutation in multiple independent colonies and the high reproducibility of the growth characteristics on plates with different (concentrations of) herbicides strongly support the fact that these mutations are involved in increased tolerance.

EXAMPLE 2

[0281] Replacing yeast ACC1 with OsJACCc60p40 dramatically reduced transformation efficiency, but allowed direct isolation of spontaneous mutants that are resistant to DIMs

[0282] RTP4107-5b-1 cells can be mutagenized by transformation of fragments carrying degeneracy in selected sites and subsequent plating on cycloxydim or tepraloxydim medium (figure 5e-5h). To determine the transformation efficiency we transformed fragments encoding the mutation I1781L to RTP4107-5b-1 and RTP4108-8b-2. In pilot experiments the efficiency was typically only 5-20 transformants/µg DNA (data not shown). The haploid strains RTP4108-8b-2 and RTP4107-5b-1 (as well as RLW001-1a-1 and RTP4106-7a-1) have a doubling time of approximately 4½ hours in liquid YPG G418 biotin FA medium. The slow growth rate and possibly altered membrane composition (Schneiter *et al.*, 1996) are believed to be the primary reasons for the poor transformation efficiencies. The transformation efficiency was ~20% higher when pre-growth of cells took place in YPG

medium supplemented with biotin and fatty acids and recovery took place in YPG medium as opposed to Clontech's standard YPD plus transformation recovery medium. Despite the improvements, transformation rates were poor, thus hampering efficient screening of high complexity mutagenized fragment mixtures.

[0283] In control experiments it was noticed that transformation of fragments not carrying any mutation resulted in the appearance of colonies on 100 and 200 μM cycloxydim and 1 and 10 μM tepraloxydim plates. Moreover, direct plating of cells on selective medium yielded resistant colonies at a frequency of 1 in ~1-5x 10^9 cells. Sequence analysis of these spontaneous mutants revealed that these colonies possessed specific mutations in the C-terminal domain of rice hybrid ACCase. Spontaneous mutations also occurred after transformation of fragments that carried mutations, like for instance I1781L. Thus in some cases the intended mutation, like in this example I1781L, was not observed, but another mutation was found in a different position. The following summarizes all spontaneous mutations obtained deliberately by direct plating of RTP4107-5b-1 or unintentionally as background in transformation experiments described below.

[0284] Table of spontaneous mutations conferring DIM resistance and the number of colonies harboring these mutations identified in all experiments. Novel mutations are indicated by *; novel mutations at novel sites by **

Mutation	Identified	Nucleotide change
I1781L	7x	ATT → CTT
I1781T*	5x	ATT → ACT
I1781V*	42x	ATT → GTT
G1783C**	1x	GGT → TGT
A1785G**	2x	GCT → GGT
A1786P**	2x	GCA → CCA
I1811N**	4x	ATT → AAT
W1999G*	57x	TGG → GGG
V2049F**	13x	GTC → TTC
W2074L**	2x	TGG → TTG
V2075G	5x	GTT → GGT
V2075I**	8x	GTT → ATT
V2075L**	99x	GTT → CTT
D2078G	142x	GAC → GGC
ΔK2080I2081**	2x	AAGATT → Δ
C2088R	35x	TGC → CGC
C2088W*	5x	TGC → TGG
G2096A	101x	GGT → GCT
G2096S*	12x	GGT → AGT
V2098A**	62x	GTT → GCT
V2098G**	66x	GTT → GGT

[0285] Among the mutations identified, we found I1781L, D2078G, C2088R and G2096A. These mutations have been known to confer resistance (Powles and Yu, 2010) and validated the yeast model system. I1781V and I1781T are novel mutations in a known site. W1999C has been reported as a mutation that allowed growth in the presence of FOPs, but not DIMs (Powles and Yu, 2010). Interestingly, we identified W1999G as a strong candidate for conferring DIM resistance. The remaining mutations in the preceding table are novel mutations at novel sites, of which I1811, V2049, V2075 and V2098 were later implied to be involved in tepraloxydim binding by studying the protein structure of yeast ACC1 (Xiang *et al.*, 2009).

[0286] The yeast model is further validated by the high frequency at which the same, novel mutations were identified (preceding table, second column). All A1785G and V2075G mutants may be dependent as they were isolated in one experiment, but all others appeared independently in two or more experiments, often after starting with the RTP4107-5b-1 and RTP4108-8b-2 strains. In a single experiment, liquid cultures of RTP4107-5b-1 were

incubated in the presence of cycloxydim to enrich the culture for cells with mutations that confer resistance to this herbicide. This alternative method for obtaining spontaneous mutants was successful, but was not followed up on because of the increased likeliness that mutants are daughter cells from a single mutation event.

[0287] Some of the herbicide resistance-conferring new amino acids in the plastidic domain turned out to correspond to residues already present in ACC1 and cytosolic plant ACCases, suggesting that these residues may partially contribute to the natural resistance of the non-plastidic forms. Isoleucine at position 1781 and alanine at position 1785 in plastidic ACCases are leucine and glycine, respectively in non-plastidic ACCase. Alignments between resistant and susceptible ACCase may be valuable in identifying potential DIM-resistance conferring residues. Apart from I1781L and A1785G we did not obtain more mutations of this type, but it should be noted that the screenings described here were not fully saturated.

[0288] Generation of DIM-resistant mutants through transformation of fragments carrying degeneracy in selected codons

[0289] Although plating of RTP4108-8b-2 and RTP4107-5b-1 is an easy and convenient method to obtain mutants, it has limitations. All mutations listed in the table of spontaneous mutations conferring DIM resistance above were single nucleotide substitutions. The spontaneous introduction of two or three mutations in a single triplet is a rare event. With single nucleotide substitutions approximately 2/3 of the amino acids will never be found at any given position. We used mutagens to increase the frequency of mutations, but such treatments led to an increased number of single nucleotide substitutions in two or more triplets throughout the 3' coding end. Despite the low efficiency, transformation of fragments with complete degeneracy at a chosen position is the best method to test all amino acids at that position. Based on pilot experiments with I1781L fragments and the average frequency of the appearance of spontaneous mutants, it was estimated that transformations had to be performed at a scale that was six times larger than the library scale described in Clontech's Yeastmaker™ Yeast Transformation System 2 kit protocol to obtain 99% certainty that all triplets were transformed.

[0290] Sites that were randomized were chosen based on results obtained in the table of spontaneous mutations conferring DIM resistance above (I1781, W1999, V2049, V2075, D2078 and V2098) and literature (W2027 and I2041). Resistant colonies isolated after transformation and plating on cycloxydim and tepraloxydim were partially sequenced

(Figures 8 and 9). Transformations were significantly "contaminated" with spontaneous mutants as anticipated. However, for most transformations we observed enrichment in the number of colonies that have mutations in the triplet that was varied (shaded cells in Figure 8). Transformations with fragments with degeneracy in W2027 and I2041 yielded only spontaneous mutants (Figure 9), consistent with the results described above.

[0291] We identified novel amino acid mutations that required more than one base pair to be altered in a single triplet. These included V2075M (GTT → ATG), D2078T (GAC → ACG and ACT), V2098H (GTT → CAC), V2098P (GTT → CCG and CCC) and V2098S (GTT → AGC, AGT and TCG). Thus the transformation method described here allowed the isolation of novel mutants which were practically impossible to obtain by relying on spontaneous mutations alone. Other methods by which novel mutants can be identified include: i. changing the codon usage of *OsJACCc60p40* in RTP4107-5b-1 to allow alternative amino acid substitutions upon spontaneous nucleotide changes. ii. exploring mutagens that alter consecutive nucleotides. iii. switching the chromosome-plasmid shuffling and mutagenesis step in Figure 5; the diploid strain with *OsJACCc60p40*, which still relies on *ACC1* for FA biosynthesis, can be made more competent for LiAc/PEG-based transformation of mutagenized fragments prior to sporulation and subsequent selection on DIM plates. Alternatively, linearized plasmids harboring *OsJACCc60p40* with small deletions can be co-transformed with mutagenized repair fragments which can be incorporated by homologous recombination repair prior to sporulation and DIM selection. These methods may increase the throughput of mutagenized fragments allowing the discovery of novel mutations in a more efficient way.

[0292] In many cases two or three different triplets were found to be mutated in a single colony (Figure 8). For instance novel V2049 variants (V2049A, V2049C, V2049L, V2049S, V2049T) always had a second D2078G or V2098A mutation. From this experiment we cannot conclude whether the novel V2049 mutations contributed to resistance or represented mutations that could be classified as "neutral" to both herbicide-tolerance and enzymatic function of the protein. The easiest way to test this is to compare growth curves of strains having V2049 mutations in combination with D2078G or V2098A to D2078G and V2098A single mutants in the presence of DIMs (see below). V2098A and V2098G mutations were often accompanied by C2088 mutations. Both the V2098 and C2088 mutations used a wide variety of codons (data not shown); thus both mutations were almost certainly introduced from the synthetic DNA fragments (unlike the double mutants involving V2049, which were

always accompanied by "single nucleotide-mutated" D2078G and V2098A residues). Epoch Biolabs confirmed that their synthetic fragment mixtures possessed imperfections due to technical limitations to the production of these fragments. V2098 mutagenesis "enriched" for imperfections at position C2088.

[0293] Control experiments with I1781L fragments that were performed in parallel resulted in the appearance of 333 colonies on cycloxydim plates. "Wt control" fragments yielded 33 colonies on the same medium (table 2 only displays the mutants that were sequenced). We calculated the level of saturation as follows:

$$\text{Saturation} = \left(1 - \left(\frac{63}{64}\right)^{(333-33)}\right) \times 100\% = 99.1\%$$

[0294] The high level of saturation is initially supported by the appearance of six V2075M mutants (Figure 8). Methionine is encoded by ATG only and the six transformants were independently obtained as the doubling time of rice ACCase-dependent yeast strains is much longer (4½ h) than the regeneration time used in the transformation procedure (1½ h). However, other statistics suggest a lack of saturation. For instance, the V2075L mutants were encoded by CTG, CTT, TTG and TTA, but never by CTC or CTA and V2075I mutants were never isolated from tepraloxydim plates, even though this mutation should confer resistance to the concentration used. We speculate that Epoch's fragment mixtures do not contain equimolar amounts of all possible variants and that our screening was not completely saturated. Thus, additional mutations in V2075, as well as in all other sites investigated here, that confer DIM resistance may still exist. These putative mutants can be uncovered by increasing the scale of the transformation experiments or by parallel transformations with fragments encoding for single, untested amino acids in selected sites.

[0295] Generation of double mutants with increased tolerance to herbicides

[0296] Double mutants can be made by transforming fragments with a DIM-resistance-conferring mutation to an acceptor strain with another DIM-resistance-conferring mutation and subsequent plating on a high DIM concentration that inhibits growth of untransformed single mutants and allows selection of superior double mutants. Figure 7 shows that growth of yeast expressing yeast ACC1 was slightly reduced by 200 μM cycloxydim. We confirmed that this effect was not caused by methanol, which was used as a solvent for cycloxydim. Therefore, we chose tepraloxydim at a concentration of 75 μM (~150x the concentration that

significantly inhibited OsJACCc60p40-dependent strains and ~8x the concentration that significantly inhibited "I1781L strains") for selection. Figure 10 shows that I1781L+V2075L, W1999G+V2075L, W1999G+D2078G, W1999G+G2096A and W1999G+V2098A mutants were obtained as intended. However, most intended double mutants were not obtained. Instead, spontaneous second mutations were often found in addition to the mutation already in place in the acceptor strain. The absence of intended double mutants could have had two causes: i. the two single mutations didn't have a synergistic or even additive effect on herbicide tolerance or compromised the function of the enzyme in a general way, ii. the transformation efficiency of rice hybrid ACCase dependent yeast was too low in some cases. All transformations that yielded the intended double mutants (indicated in shaded squares in Figure 10) were obtained in a single transformation experiment. All other transformations shown in Figure 10 were attempted four times without success. Some mutations may not work well together, but additional transformations are required to acquire more certainty.

[0297] Double mutants involving residues that are close together (e.g. G2096A and V2098A) can be obtained by transforming RTP4107-5b-1 with a fragment carrying both mutations. This strategy was not used here. Instead, we reasoned that those and other double mutants could be obtained much easier by plating single mutants on plates with 75 µM tepraloxydim and waiting for spontaneous second mutations to appear. This method is not only less labor-intensive, it also automatically selects for mutations that perform well together. Figure 11 shows the single mutants that were plated (between 1.5×10^9 and 5×10^9 cells), including the mutants that required more than one nucleotide change (V2075M, D2078T, V2098H, V2098P, V2098S), and the additional, spontaneous mutations that were gained. In this table the frequency of the appearance of these mutations is less relevant, because the double mutants could be daughter cells from a single event.

[0298] Growth curves of mutants in the presence of tepraloxydim, cycloxydim and haloxyfop

[0299] The majority of the single and double mutants isolated here were grown in liquid cultures with and without herbicides to obtain growth curves and compare their relative effectiveness. Next to cycloxydim and tepraloxydim we were interested in the growth characteristics of mutants in the presence of haloxyfop, which belongs to the FOP class of herbicides. We made "kill curves" on solid haloxyfop plates first with RTP4107-5b-1 and DIVE292, a strain that possessed the I1781L mutation (Figure 7C). The activity of haloxyfop

was comparable to that of cycloxydim. All methods described here can also be applied to FOP herbicides.

[0300] Growth curves were made with 100 and 200 μM cycloxydim, 2, 10 and 100 μM tepraloxydim, 100 and 200 μM haloxyfop and without herbicide (see Figures). In all previous experiments we used cycloxydim from Sigma-Aldrich dissolved in methanol. However, the product got discontinued, so the final growth curves were made with cycloxydim formulation, dissolved in naphta. The formulation has a negative effect on the yeast strain that depends on ACC1 for FA biosynthesis during the first 2 days of growth (Figure 12). This phenomenon was not observed in earlier experiments with cycloxydim dissolved in methanol. We believe that naphta delays growth, but after evaporation of the solvent growth is quickly restored. The rice hybrid ACCase-dependent strains grow much slower and naphta effects were not clearly visible. Pilot growth curve experiments with cycloxydim dissolved in methanol confirmed this view for all mutants tested as graphs were found to be highly similar to the graphs presented here (data not shown).

[0301] The OsJACCc60p40 control strain (RTP4107-5b-1) was unable to grow in the presence of any of the herbicides tested here (Figure 13). All single mutants showed a certain degree of herbicide tolerance. The single mutants W1999G and V2098A were tested in triplicate with three independently obtained strains (Figures 14-19). The triplicates produced similar graphs. However, caution should be taken comparing acrossdifferent strains. We aimed at starting all cultures with the same amount of cells, but the general fitness of cultures may differ depending on how long pre-cultures were in stationary phase prior to dilution into medium with herbicides. Growth should always be judged as relative growth compared to growth in medium without herbicide. The best single mutations conferring DIM resistance are D2078G, V2098A and V2098G. V2075M (and to a lesser extent V2075L) is a good mutation for growth in tepraloxydim medium, but not cycloxydim medium. This mutation has potential to be used in rotation with the I1781L mutation, for which it has been shown that it provides commercial tolerance to cycloxydim, but not tepraloxydim. Haloxyfop resistance is most pronounced in W1999G and C2088R mutants.

[0302] We isolated a large number of double mutants in which one mutation was never characterized as a single mutation. Therefore these mutations may not have contributed to resistance. The following table shows the relative change in tolerance to cycloxydim,

tepraloxydim and haloxyfop due to the second, uncharacterized mutation as compared to the characterized mutation.

[0303] Table of changes in relative resistance to cycloxydim, tepraloxydim and haloxyfop between single mutants that have been fully characterized and double mutants that have an untested mutation in combination with the tested mutations listed.

untested	tested	cycloxydim	tepraloxydim	haloxyfop
S1792L	V2049F	ND	ND	ND
Q1824P	D2078G	—	—	—
Q1824P	I1781L	—	—	—
A1837V	V2075I	↑↑	↑↑	↑↑
V1864F	I1781L	—	↑↑	↓↓ (gone)
V1864F	W1999G	↓↓ (gone)	↑↑	↓↓ (gone)
W1999C	V2075I	↑↑	↑↑	↑↑
W1999L	V2049F	ND	ND	ND
W2027R	I1781L	↓	↓↓	↓
E2039G	D2078G	—	—	—
I2041V	D2078G	↑↑	↑↑	↑↑
V2049A	D2078G	ND	ND	ND
V2049C	D2078G	↑	↑	↓↓ (gone)
V2049I	W1999G	—	↑↑	—
V2049L	V2098A	↓↓	↓↓	↓↓ (gone)
V2049S	D2078G	ND	ND	ND
V2049T	D2078G	↓	↑↑	↓↓ (gone)
dupIV2075	I1781L	—	↑↑	—
S2079F	D2078G	↓↓	↓↓	↓↓
S2079P	D2078G	↓	↓	↓
K2080E	V2075M	— (none)	↑↑	↑↑
C2088F	V2049F	ND	ND	ND
C2088F	V2098A	↓	↓	↓
C2088G	D2078G	—	↑↑	↑
C2088G	V2098G	↓	—	— (none)
C2088H	V2098A	↑↑	↑↑	↑
C2088H	V2098G	—	↑↑	↑
C2088K	V2098A	↑↑	—	—
C2088L	V2098A	↑↑	—	↑
C2088L	V2098G	↓	↑	— (none)
C2088S	V2098G	↓	—	— (none)
C2088T	V2098A	ND	ND	ND
C2088T	V2098G	—	↑↑	— (none)
C2088V	V2098G	↓	↑	↑
K2095E	V2075M	— (none)	↑↑	—
V2098C	C2088W	↓↓	↑	— (none)

Grey boxes represent double mutants picked up from high concentration tepraloxydim plates (Figures 10 and 11). ND = Not Determined; — = no change in tolerance; ↓ = decrease in tolerance; ↑ = increase in tolerance.

[0304] Mutations Q1824P and E2039G did not seem to change herbicide tolerance and other mutations had a negative effect on herbicide tolerance (W2027R, V2049L, S2079F, S2079P, C2088F and C2088S). Mutations A1837V, V1864F, W1999C, I2041V, V2049C, V2049I, V2049T, duplication V2075, K2080E, C2088G, C2088H, C2088K, C2088L, C2088T, C2088V, K2095E and V2098C increased tolerance to at least one herbicide. In some cases there was an accompanying decrease in tolerance to (an)other herbicide(s) (V1864F, V2049C, V2049T, C2088L, C2088V and V2098C). Most notably the extra presence of V1864F completely eliminated the high tolerance that I1781L and W1999G single mutants displayed for haloxyfop. Such observations are important in light of possible rotation between DIMs or between DIMs and FOPs in cultivated, modified, non-GMO rice in the future. All isolations described here were selected on DIM plates. The V2075G mutant, which was isolated from cycloxydim plates, is the most "FOP-specific" mutation that was isolated. Mutants that are specifically tolerant to FOPs can be obtained by repeating all screenings described here and selecting on FOP plates.

[0305] Among the double mutants consisting of two separately-characterized single mutations, I1781L+V2075L, W1999G+V2075L and W1999G+V2098A were found to be fully resistant to ~150-200x the tepraloxydim concentration that completely halts growth of the OsJACCc60p40 control. These three double mutations were also highly resistant to cycloxydim and haloxyfop. Growth curves were made with three independently obtained strains (see Figures) and results were highly reproducible in multiple experiments (data not shown). These three double mutations, together with the V2075M single mutation, have been introduced in fully plastidic rice ACCase and can be used to generate transgenic plants, for example, transgenic corn. A1837V+V2075I, I1781L+V2049F, I1781L+V2098G, W1999G+D2078G, W1999C+V2075I and I2041V+D2078G may be also be used.

[0306] Additional double mutants that may be used include, but are not limited to, S1792L+V2049F, N1780D+I1781L, I1781V+V2049F, A1785G+D2078G, A1785G+V2098A, A1785G+V2098G, W1999L+V2049F, V2049A+D2078G, V2049F+C2088F, V2049S+D2078G, A2059G+D2078G, V2075L+C2088W and C2088T+V2098A.

[0307] The single mutants V2075L, V2098A and V2098G are the most tepraloxydim resistant single mutants, with V2075L exhibiting lesser tolerance to cycloxydim and V2098G exhibiting more. Double mutants containing W1999G in combination with I1781L and/or

V2075L exhibited good tolerance. The double mutant W1999G V2075L is resistant to 100 μM tepraloxydim (compare to I1781L T100). W1999C appears to specifically provide tolerance to only FOPs.

[0308] References

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[0327] Whereas, particular embodiments of the invention have been described above for purposes of description, it will be appreciated by those skilled in the art that numerous variations of the details may be made without departing from the invention as described in the appended claims.

[0328] All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

CLAIMS

We claim:

1. A method of producing an acetyl-CoA carboxylase (ACCase) enzyme that is tolerant to at least one herbicide, comprising:

a. providing an ACCase-deficient yeast that comprises a nucleic acid encoding a chimeric ACCase, said chimeric ACCase comprising an N-terminal region and a C-terminal region, wherein the C-terminal region comprises an herbicide sensitivity region,

b. contacting said yeast with at least one mutagenic oligonucleotide under conditions that permit site-directed mutagenesis of at least one codon of the nucleic acid encoding the chimeric ACCase, and growing the mutagenized yeast thereby forming a library of mutagenized yeast colonies;

c. culturing the mutagenized yeast colonies in the presence of at least one ACCase-inhibiting herbicide to form treated colonies; and

d. isolating from at least one of said treated colonies at least one mutagenized yeast that grows in the presence of the herbicide, wherein the mutagenized yeast that grows in the presence of the herbicide comprises a mutagenized ACCase that has a tolerance to the herbicide that is greater than that exhibited by the chimeric ACCase of step (a),

wherein the method is a high throughput method.

2. The method according to claim 1, wherein the nucleic acid encoding the chimeric ACCase is a single copy plasmid.

3. The method according to claim 1, wherein the oligonucleotide comprises more than one nucleotide mismatch with the sequence of the nucleic acid encoding the chimeric ACCase resulting in mutation of more than one base of the nucleic acid.

4. The method according to claim 1, wherein the oligonucleotide is a library of oligonucleotides with differing sequences.

5. The method according to claim 4, wherein the library comprises oligonucleotides having all possible codons at a position coding for an amino acid in the herbicide sensitivity region.

6. The method according to claim 1, wherein the herbicide sensitivity region comprises an amino acid sequence of a monocot plastidic ACCase corresponding to an amino acid

sequence selected from the group consisting of 1654(*Am*) to 2204(*Am*), 1654(*Am*) to 2130(*Am*), 1654(*Am*) to 2098(*Am*), 1750(*Am*) to 2204(*Am*), 1750(*Am*) to 2130(*Am*), 1750(*Am*) to 2098(*Am*), 1781(*Am*) to 2204(*Am*), 1781(*Am*) to 2130(*Am*), or 1781(*Am*) to 2098(*Am*).

7. The method according to claim 6, wherein the monocot is an *Oryza* species.

8. The method according to claim 1, wherein the herbicide sensitivity comprises at least one herbicide tolerance mutation prior to contacting with the mutagenic oligonucleotide.

9. The method of claim 1, wherein said ACCase-deficient yeast comprises a mutation of the genomic yeast ACCase gene selected from the group consisting of: a single point mutation, multiple point mutations, a partial deletion, a partial knockout, a complete deletion and a complete knockout.

10. The method of claim 1, wherein said N-terminal region is derived from a yeast or fungi genus selected from the group consisting of *Saccharomyces, Schizosaccharomyces, Candida, Ascomycetes Neurospora, Kluyveromyces, Picha, Cryptococcus, Chrysosporium, Yarrowia, Arxula,* and *Hansenula.*

11. The method of claim 1, wherein said N-terminal region or C-terminal region is derived from a monocot genus selected from the group consisting of *Saccharum*, *Poa*, *Agrostis*, *Lolium*, *Festuca*, *Zoysia*, *Cynodon*, *Stenotaphrum*, *Paspalum*, *Eremochloa*, *Axonopus*, *Bouteloua*, *Arundinaria*, *Bambusa*, *Chusquea*, *Guadua*, *Shibataea*, *Erharta*, *Leersia*, *Microlaena*, *Oryza*, *Zizania*, *Triticeae*, *Aveneae*, *Hordeum*, *Lolium*, *Digitaria*, *Cyperus*, *Kyllinga*, *Erigeron*, *Hydrocotyle*, *Kummerowia*, *Euphorbia*, and *Viola, Zea, Sorghum, Pennisetum, Panicum, Setaria, Eleusine, Ananas,* and *Musa*.

12. The method of claim 1, wherein said N-terminal region is derived from a cytoplasmic ACCase from an *Oryza* species.

13. The method of claim 1, wherein said C-terminal region HSR is derived from a plastidic ACCase from an *Oryza* species.

14. The method of claim 1, wherein said herbicide is a aryloxyphenoxyproprionate (FOP) or cyclohexadione (DIM) herbicide.

15. The method of claim 14, wherein said FOP herbicide is selected from the group consisting of cyhalofop, quizalofop, diclofop, clodinafop, fluazifop, metamifop, propaquizafop, and fenoxyprop.

16. The method of claim 14, wherein said DIM herbicide is selected from the group consisting of alloxydim, butroxydim, clethodim, cycloxydim, tepraloxydim, sethoxydim, tralkoxydim, and profoxydim.

17. The method of claim 1, wherein said herbicide is present at a concentration from about 0.02 μM to about 200 μM.

18. The method of claim 1, wherein said C-terminal region comprises about 50% to about 60% of the chimeric ACCase.

19. The method of claim 1, wherein yeast cells are cultured at a temperature of about 23°C to about 30°C.

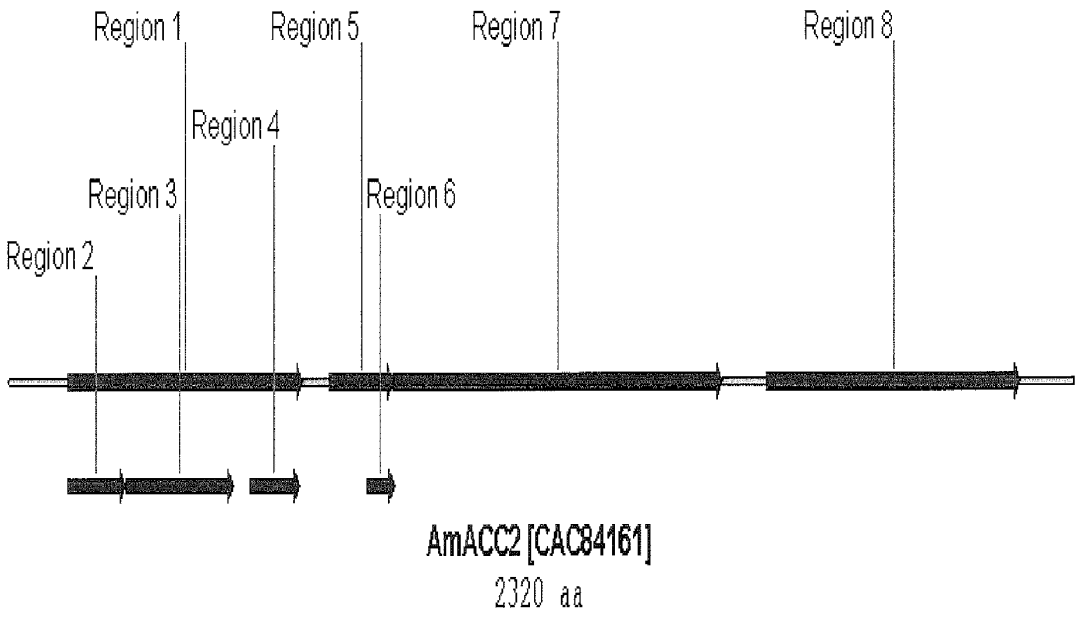
20. The method of claim 1, wherein yeast cells are cultured in liquid media.

21. The method of claim 1, wherein yeast cells are cultured on solid phase media.

ABSTRACT

The present invention relates compositions and methods for identifying, isolating, and characterizing herbicide tolerant mutations in monocot plastidic acetyl-CoA carboxylases using a model system.

FIGURE 1A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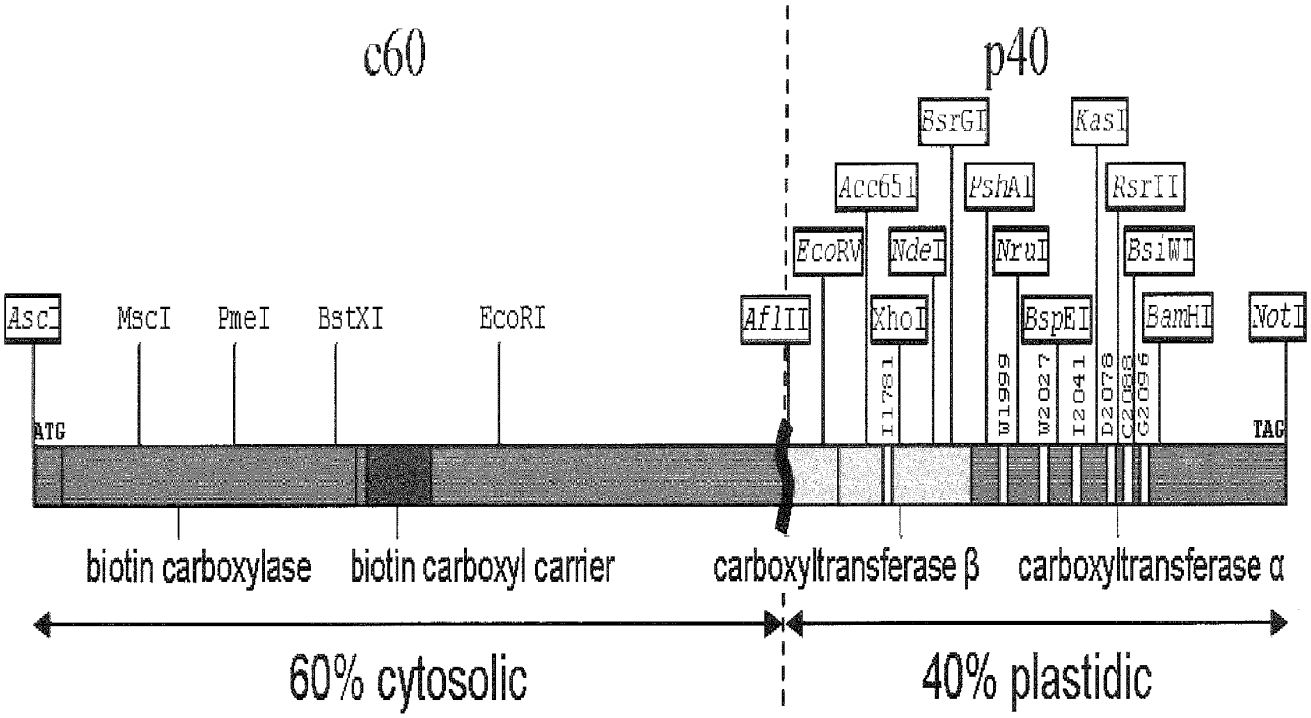
Region 5
Region 7
Region 8
Region 4
Region 3
Region 6
Region 2
AmACC2 [CAC84161]
2320 aa

FIGURE 1B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p40
BsrGI
KasI
Acc65I
PshAI
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EcoRV
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NruI
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AscI
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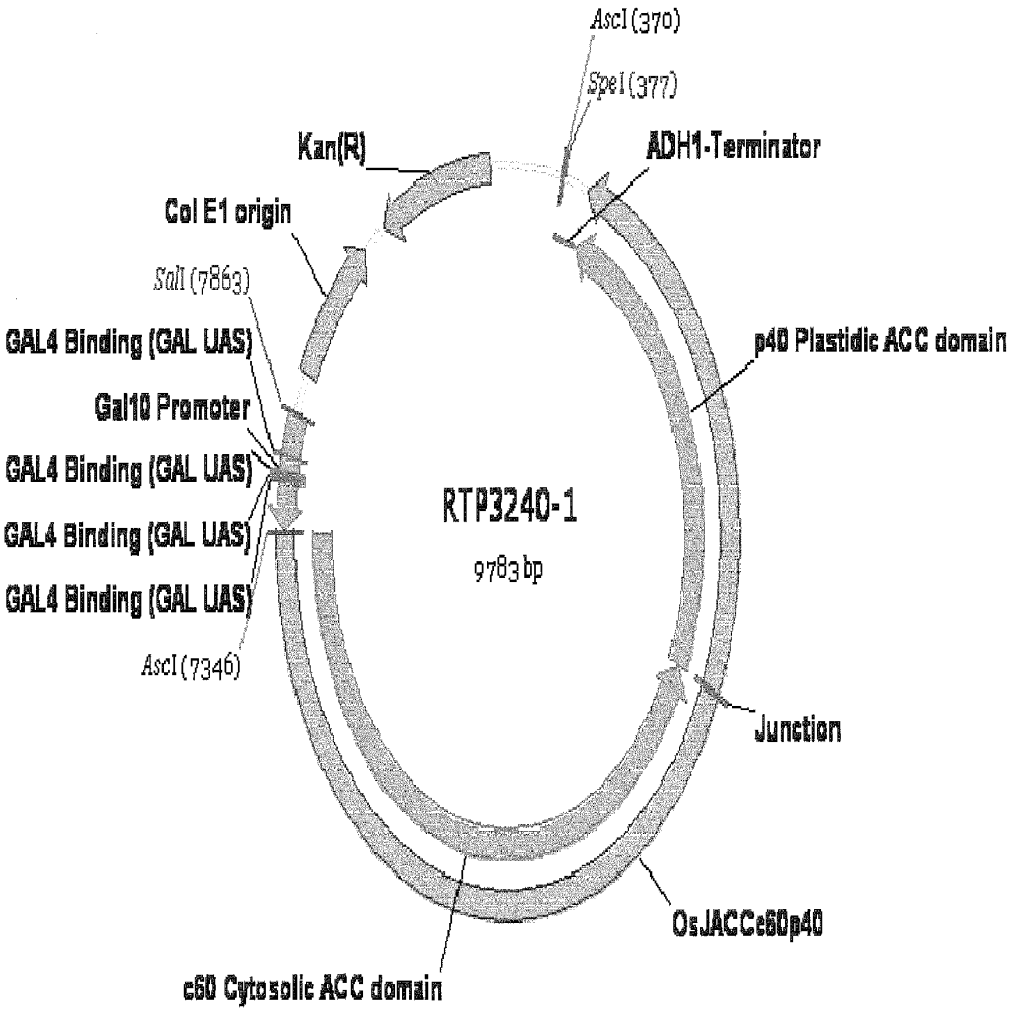
BstXI
EcoRI
AflII
XhoI
BspEI
BamHI
NotI
I1781
M1999
W2027
I2041
D2078
C2083
G2096
ATG
TAG
biotin carboxylase
biotin carboxyl carrier
carboxyltransferase β
carboxyltransferase α
60% cytosolic
40% plastidic

FIGURE 2A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SpeI (377)
Kan(R)
ADH1-Terminator
Col E1 origin
SalI (7863)
GAL4 Binding (GAL UAS)
p40 Plastidic ACC domain
Gal10 Promoter
GAL4 Binding (GAL UAS)
RTP3240-1
9783 bp
GAL4 Binding (GAL UAS)
GAL4 Binding (GAL UAS)
AscI (7346)
Junction
OsJACCc60p40
c60 Cytosolic ACC domain

FIGURE 2B

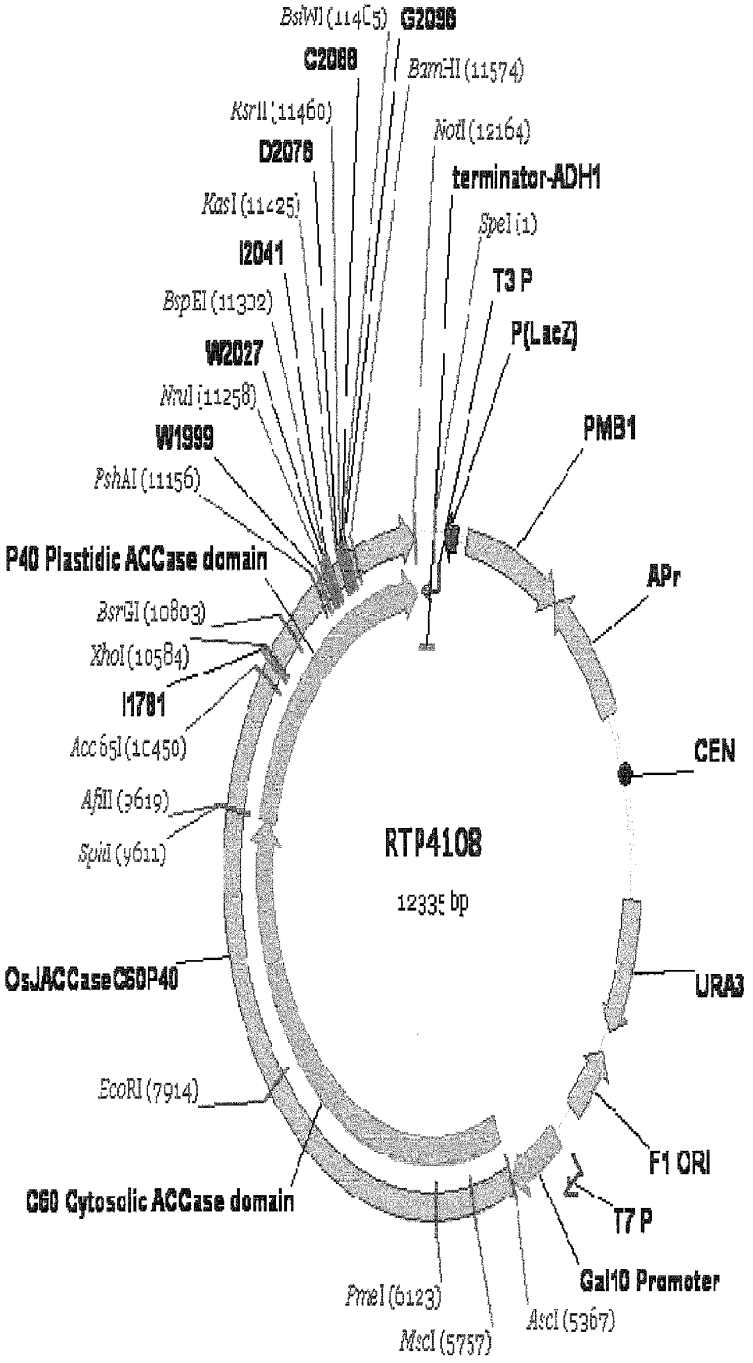
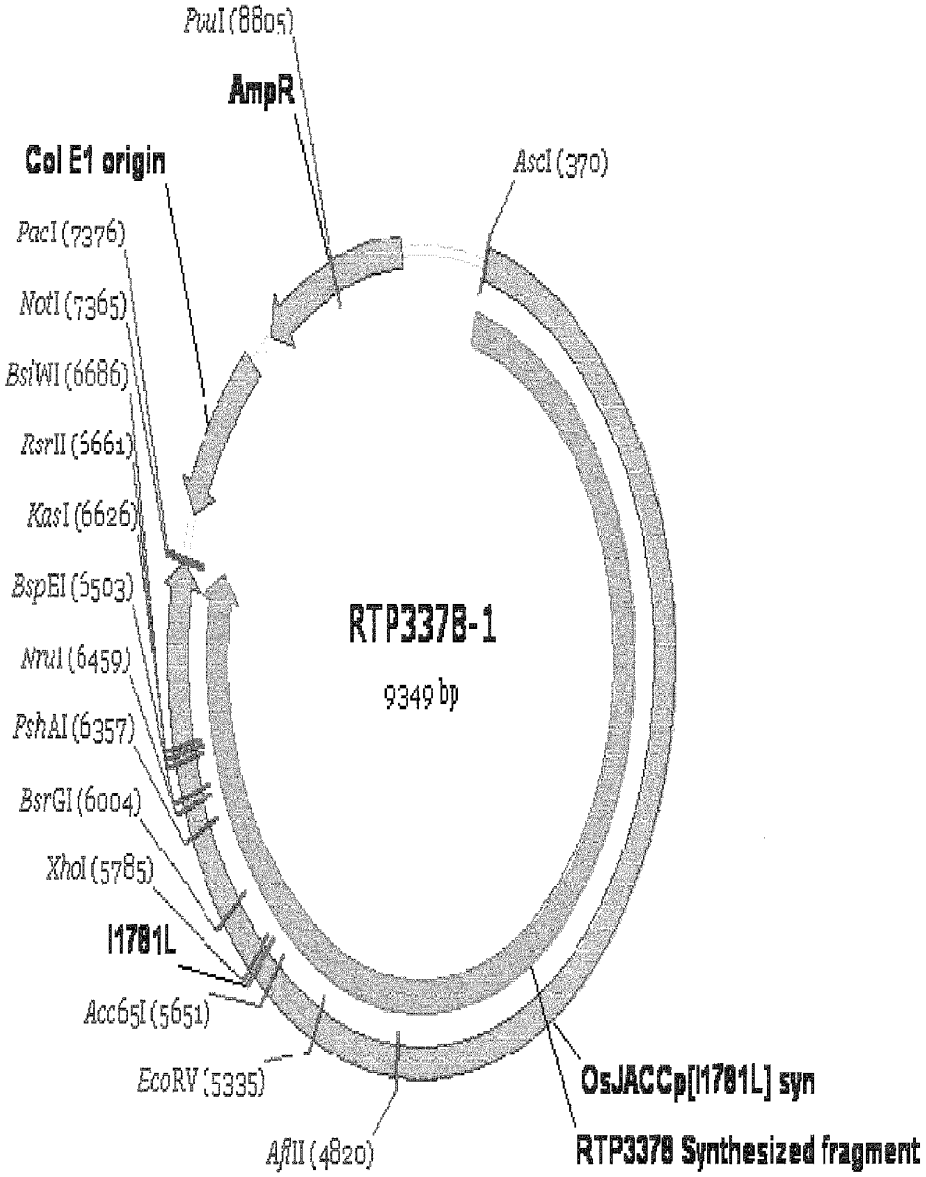

BsiWI (11405)
G2098
C2088
BamHI (11574)
KsrII (11460)
NotI (12164)
D2078
terminator-ADH1
KasI (11425)
SpeI (1)
I2041
T3 P
BspEI (11332)
P(LacZ)
W2027
NruI (11258)
PMB1
W1999
PshAI (11156)
P40 Plastidic ACCase domain
APr
BsrGI (10803)
XhoI (10584)
I1781
Acc65I (10450)
CEN
AflII (9619)
SphI (9611)
RTP4108
12335 bp
OsJACCaseC60P40
URA3
EcoRI (7914)
F1 ORI
C60 Cytosolic ACCase domain
T7 P
Gal10 Promoter
PmeI (6123)
AscI (5367)
MscI (5757)

FIGURE 2C

FIGURE 3A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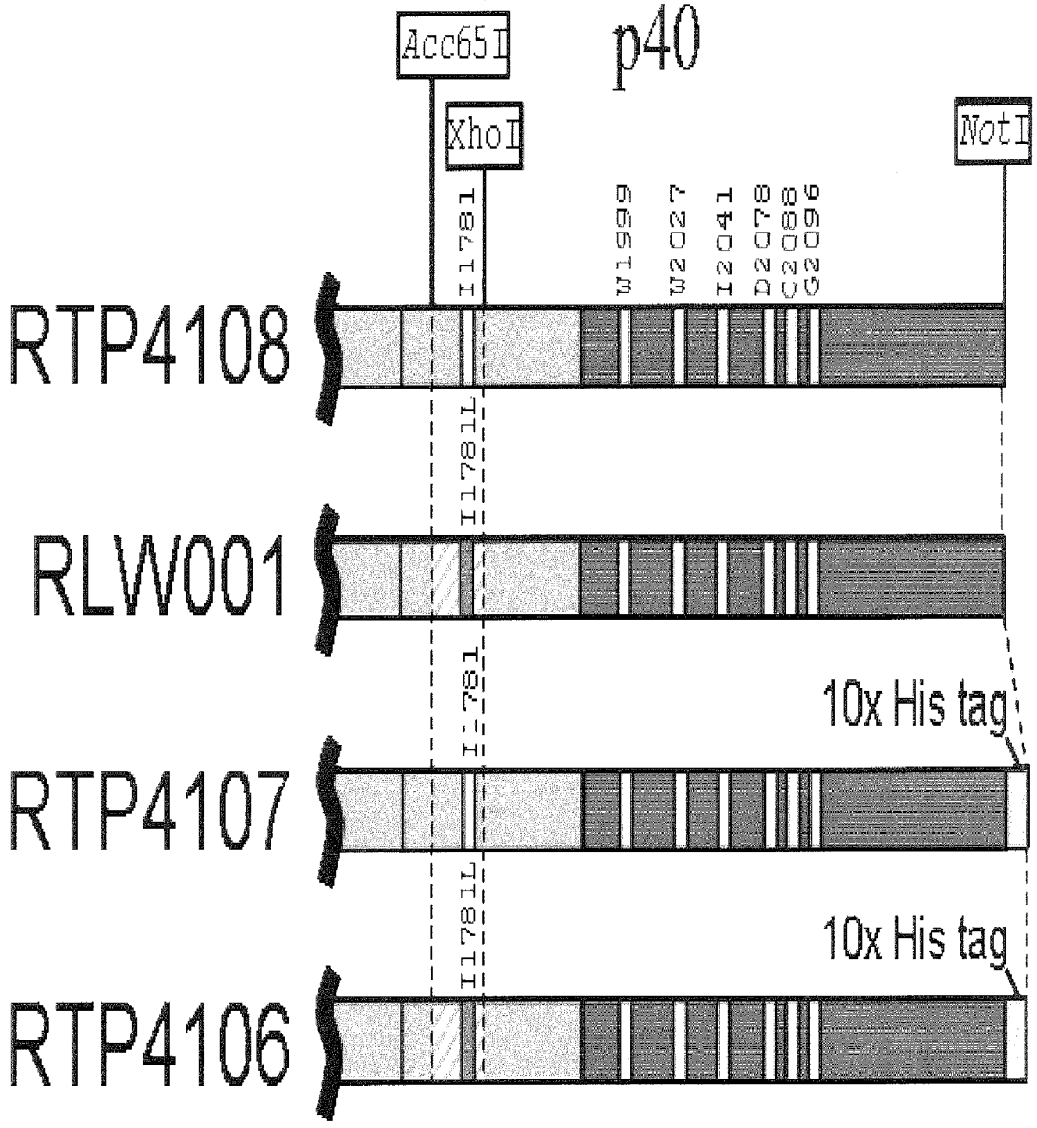
KasI (6626)
BspEI (6503)
NruI (6459)
PshAI (6357)
BsrGI (6004)
XhoI (5785)
I1781L
Acc65I (5651)
EcoRV (5335)
AflII (4820)
RTP3378-1
9349 bp
OsJACCp[I1781L] syn
RTP3378 Synthesized fragment

FIGURE 3B

FIGURE 4


Acc65I
p40
XhoI
NotI
I1781
W1599
W2027
I2041
D2078
C2088
C2096
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I1781L
RLW001
I1781
10x His tag
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I1781L
10x His tag
RTP4106

FIGURE 5

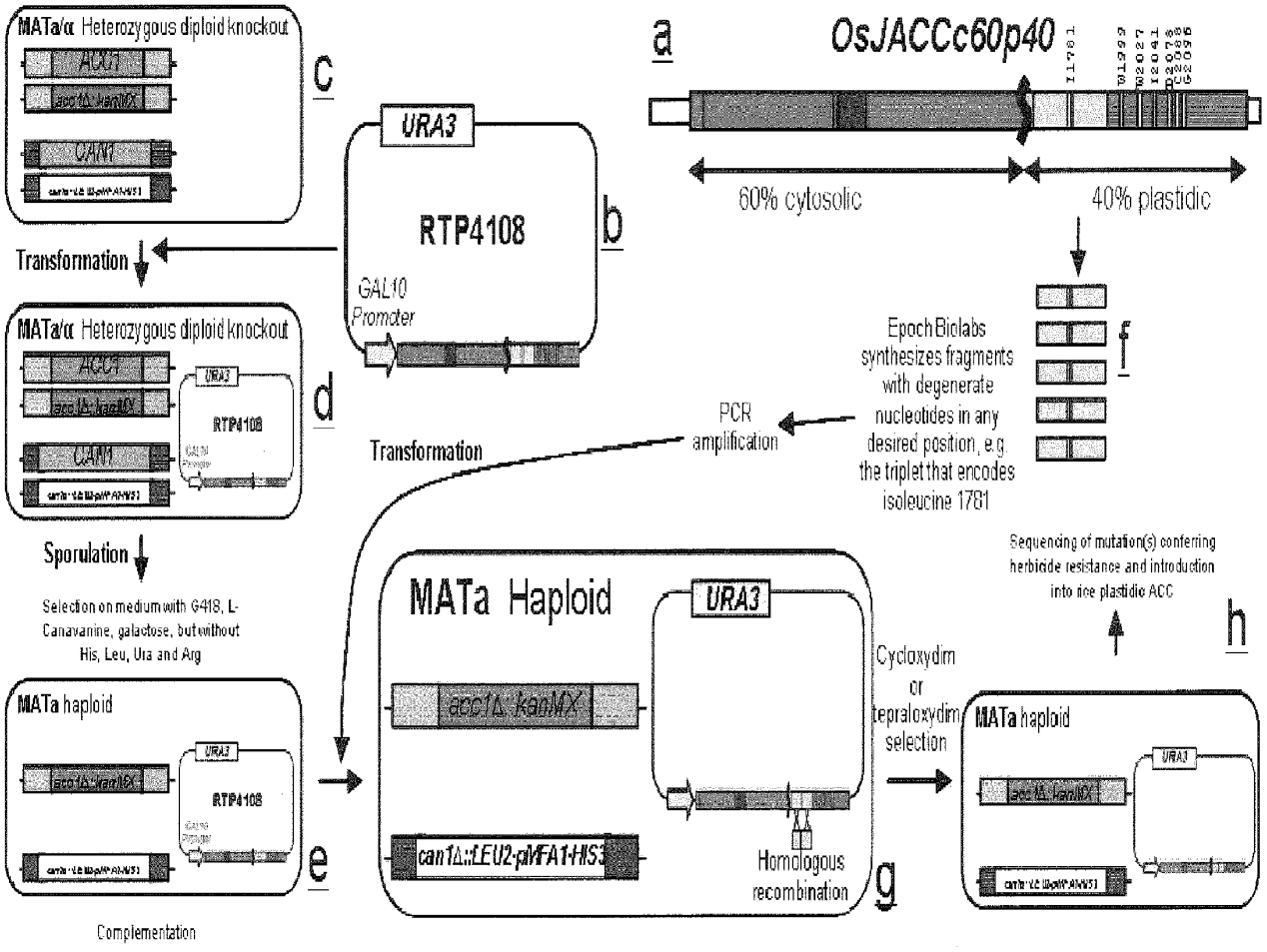

MATa/α Heterozygous diploid knockout
ACC1
acc1Δ::kanMX
CAN1
c
URA3
RTP4108
GAL10
Promoter
b
a
OsJACCc60p40
60% cytosolic
40% plastidic
Transformation
MATa/α Heterozygous diploid knockout
d
Epoch Biolabs synthesizes fragments with degenerate nucleotides in any desired position, e.g. the triplet that encodes isoleucine 1781
f
PCR amplification
Transformation
Sporulation
Selection on medium with G418, L-Canavanine, galactose, but without His, Leu, Ura and Arg
MATa haploid
e
MATa Haploid
acc1Δ::kanMX
can1Δ::LEU2-pMFA1-HIS3
Homologous recombination
g
Cycloxydim or tepraloxydim selection
Sequencing of mutation(s) conferring herbicide resistance and introduction into rice plastidic ACC
h
Complementation

FIGURE 6

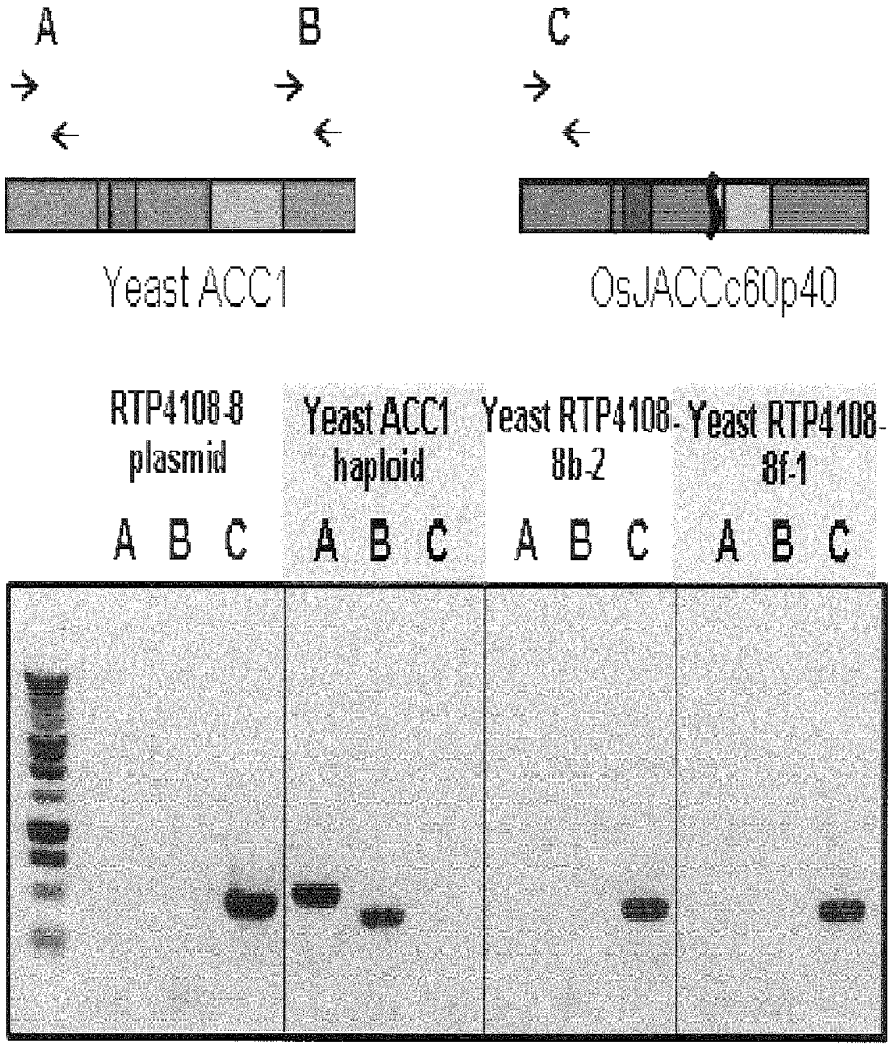
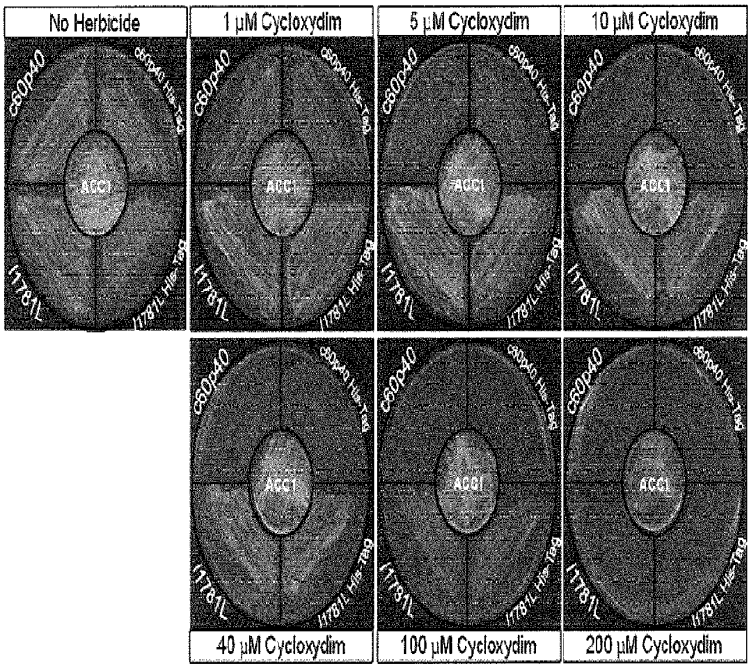

A
B
C
Yeast ACC1
OsJACCc60p40
RTP4108-8 plasmid
Yeast ACC1 haploid
Yeast RTP4108-8b-2
Yeast RTP4108-8f-1
A B C
A B C
A B C
A B C

FIGURE 7

A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1 µM Cycloxydim
5 µM Cycloxydim
10 µM Cycloxydim
40 µM Cycloxydim
100 µM Cycloxydim
200 µM Cycloxydim

B

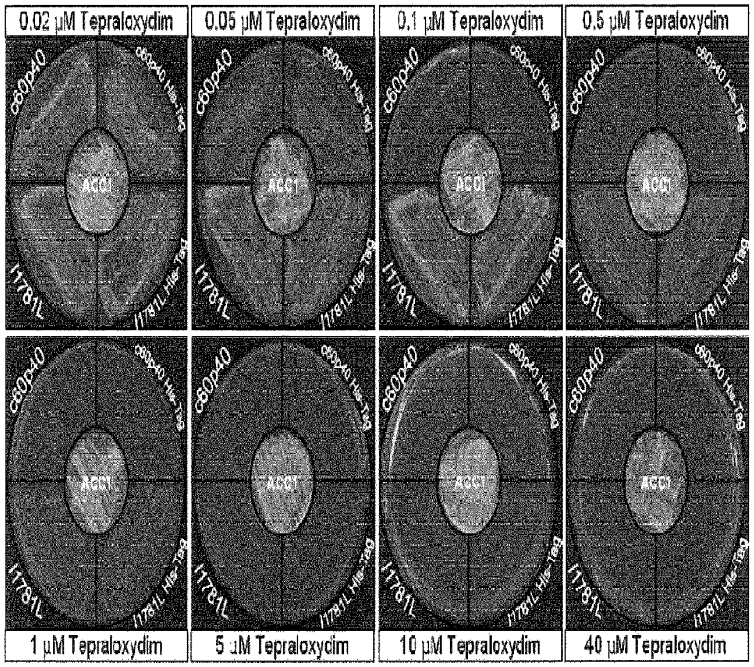

0.02 µM Tepraloxydim
0.05 µM Tepraloxydim
0.1 µM Tepraloxydim
0.5 µM Tepraloxydim
1 µM Tepraloxydim
5 µM Tepraloxycim
10 µM Tepraloxydim
40 µM Tepraloxydim

FIGURE 7C

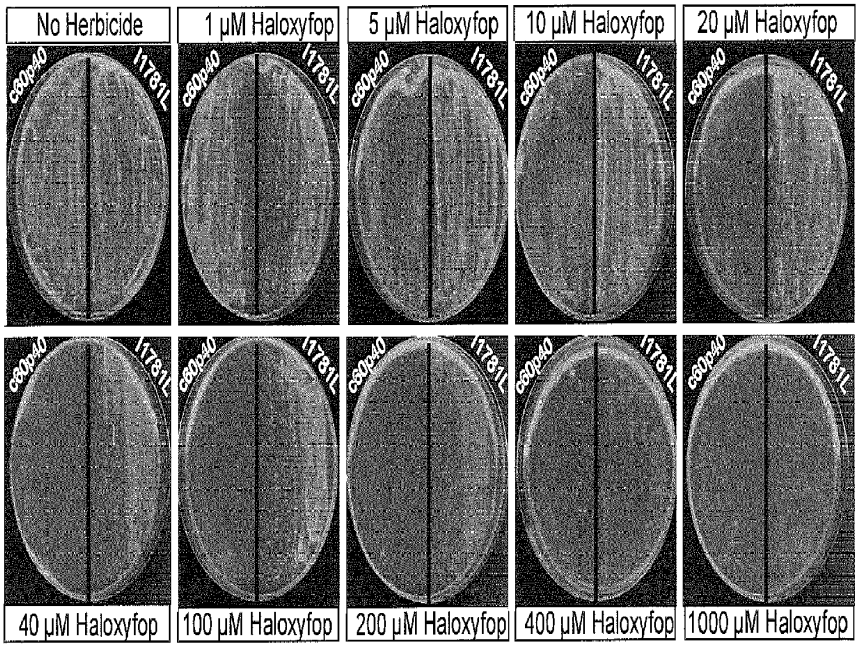

No Herbicide
1 µM Haloxyfop
5 µM Haloxyfop
10 µM Haloxyfop
20 µM Haloxyfop
c80p40
I1781L
40 µM Haloxyfop
100 µM Haloxyfop
200 µM Haloxyfop
400 µM Haloxyfop
1000 µM Haloxyfop

FIGURE 8

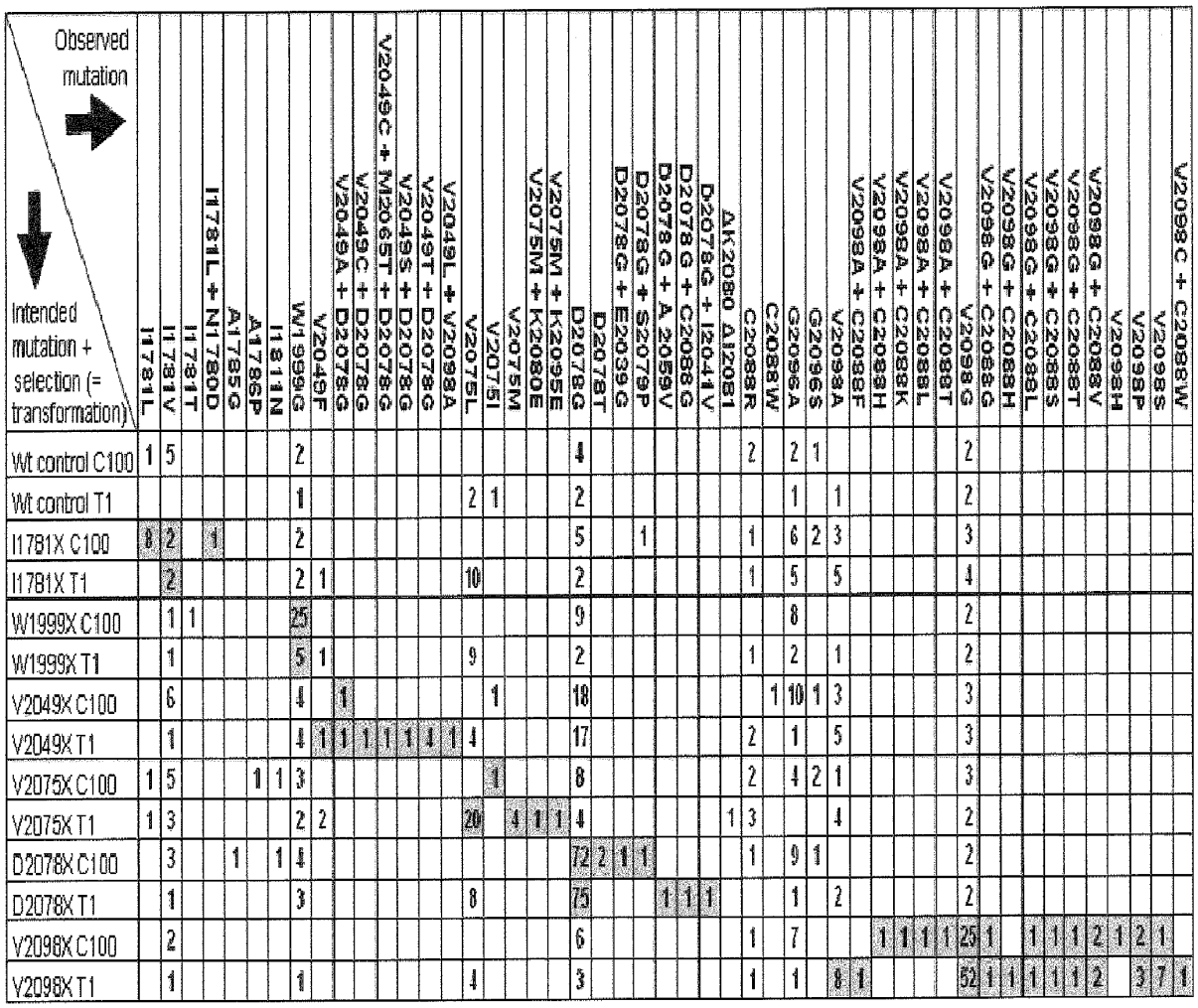
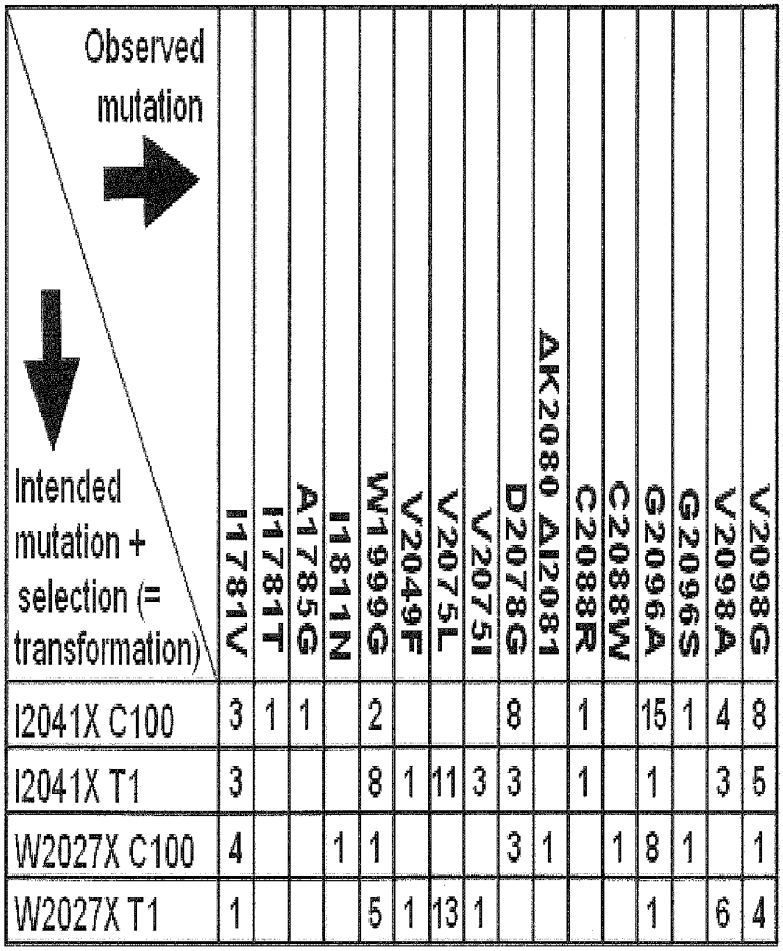

Observed mutation
Intended mutation + selection (= transformation)
I1781L
I1781V
I1781T
I1781L + N1780D
A1785G
A1786P
I1811N
W1999G
V2049F
V2049A + D2078G
V2049C + D2078G
V2049C + M2065T + D2078G
V2049S + D2078G
V2049T + D2078G
V2049L + V2098A
V2075L
V2075I
V2075M
V2075M + K2095E
V2075M + K2080E
D2078T
D2078G
D2078G + E2039G
D2078G + S2079P
D2078G + A 2059V
D2078G + C2088G
D2078G + I2041V
ΔK2080 ΔI2081
C2088R
C2088W
G2096A
G2096S
V2098A
V2098A + C2088F
V2098A + C2088H
V2098A + C2088K
V2098A + C2088L
V2098A + C2088T
V2098G
V2098G + C2088G
V2098G + C2088H
V2098G + C2088L
V2098G + C2088S
V2098G + C2088T
V2098G + C2088V
V2098G + C2088W
V2098H
V2098P
V2098S
Wt control C100
Wt control T1
I1781X C100
I1781X T1
W1999X C100
W1999X T1
V2049X C100
V2049X T1
V2075X C100
V2075X T1
D2078X C100
D2078X T1
V2098X C100
V2098X T1

FIGURE 9



Intended mutation + selection (= transformation) \ Observed mutation	I1781V	I1781T	A1785G	I1811N	W1999G	V2049F	V2075L	V2075I	D2078G	ΔK2080 ΔI2081	C2088R	C2088W	G2096A	G2096S	V2098A	V2098G
I2041X C100	3	1	1		2				8		1		15	1	4	8
I2041X T1	3				8	1	11	3	3		1		1		3	5
W2027X C100	4			1	1				3	1		1	8	1		1
W2027X T1	1				5	1	13	1					1		6	4

FIGURE 10

Transformation: acceptor	Transformation: donor	I1781L V2049F	I1781L V2075L	I1781L E2068A V2075L	I1781L dupIV2075	V1864F W1999G	W1999G V2075I	W1999G V2075L	W1999G D2078G	W1999G G2096A	W1999G V2098A
I1781L	W2027C				1						
I1781L	I2041II										
I1781L	V2075L		30	1							
I1781L	G2096A										
I1781L	V2098G	2									
W1999G	I1781L							1			1
W1999G	W2027C										
W1999G	W2027R										
W1999G	I2041II										
W1999G	V2049F						1				
W1999G	V2075L							9			1
W1999G	D2078G								6		
W1999G	C2088R					1		1			
W1999G	G2096A									1	
W1999G	V2098A										1
W1999G	V2098G						1				
D2078G	I1781L										
C2088R	I1781L										
C2088R	V2049F										
V2098A	I1781L										
V2098A	V2049F										
V2098A	V2075L										
V2098G	V2049F										
V2098G	V2075L										

FIGURE 11

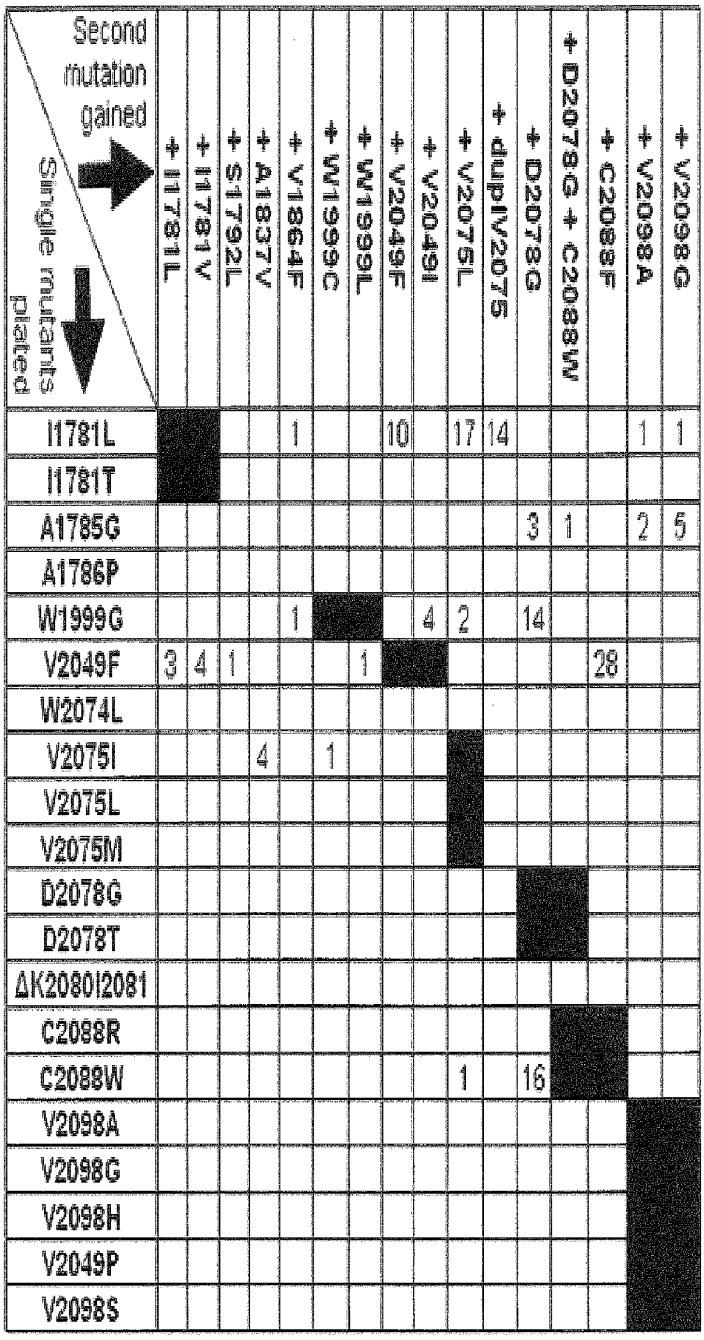

Second mutation gained
Single mutants plated
+ I1781L
+ I1781V
+ S1792L
+ A1837V
+ V1864F
+ W1999C
+ W1999L
+ V2049F
+ V2049I
+ V2075L
+ dupIV2075
+ D2078G
+ D2078G + C2088W
+ C2088F
+ V2098A
+ V2098G
I1781L
I1781T
A1785G
A1786P
W1999G
V2049F
W2074L
V2075I
V2075L
V2075M
D2078G
D2078T
ΔK2080I2081
C2088R
C2088W
V2098A
V2098G
V2098H
V2049P
V2098S

FIGURE 12

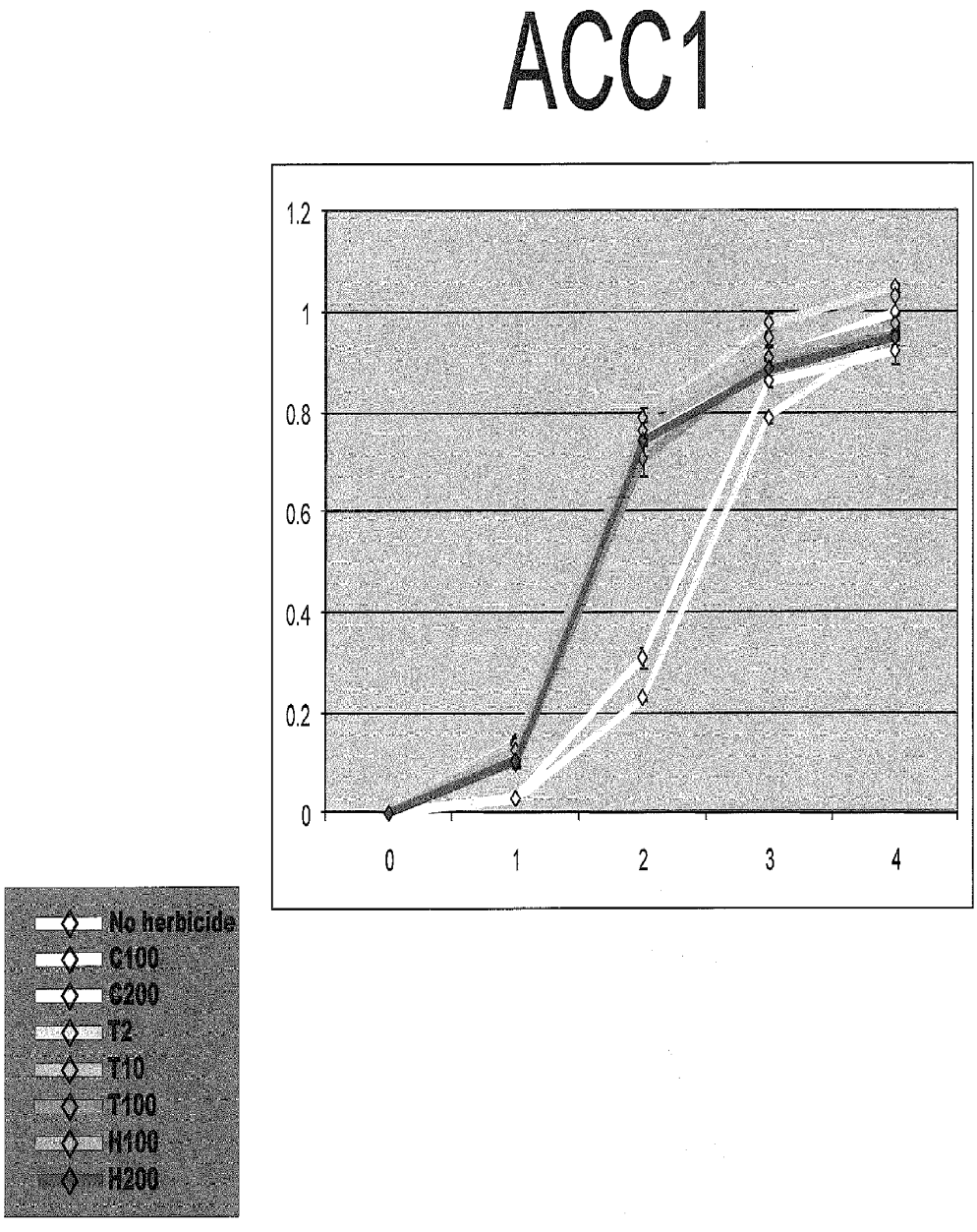

ACC1
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
No herbicide
C100
C200
T2
T10
T100
H100
H200

FIGURE 13

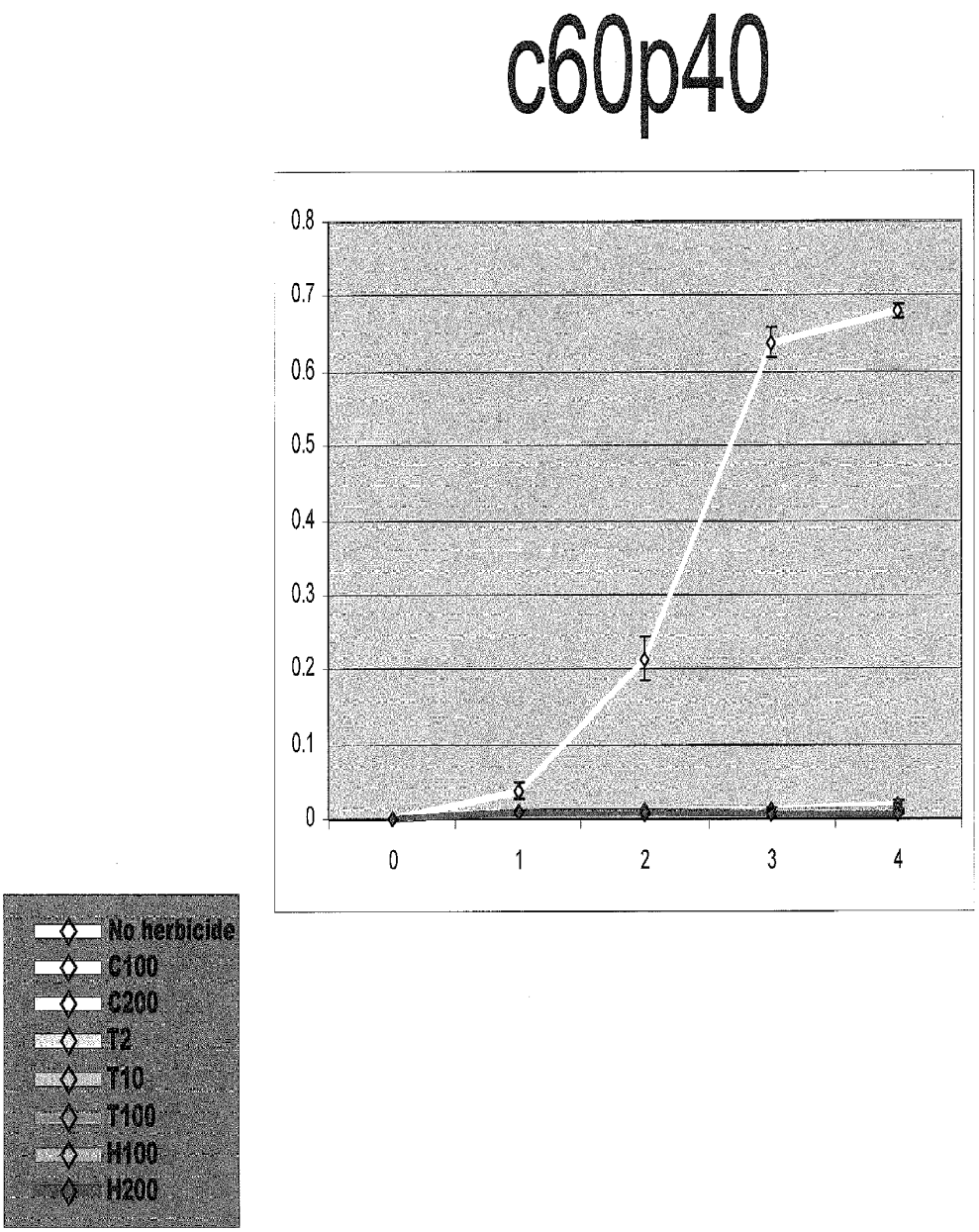

c60p40
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
No herbicide
C100
C200
T2
T10
T100
H100
H200

FIGURE 14

W1999G

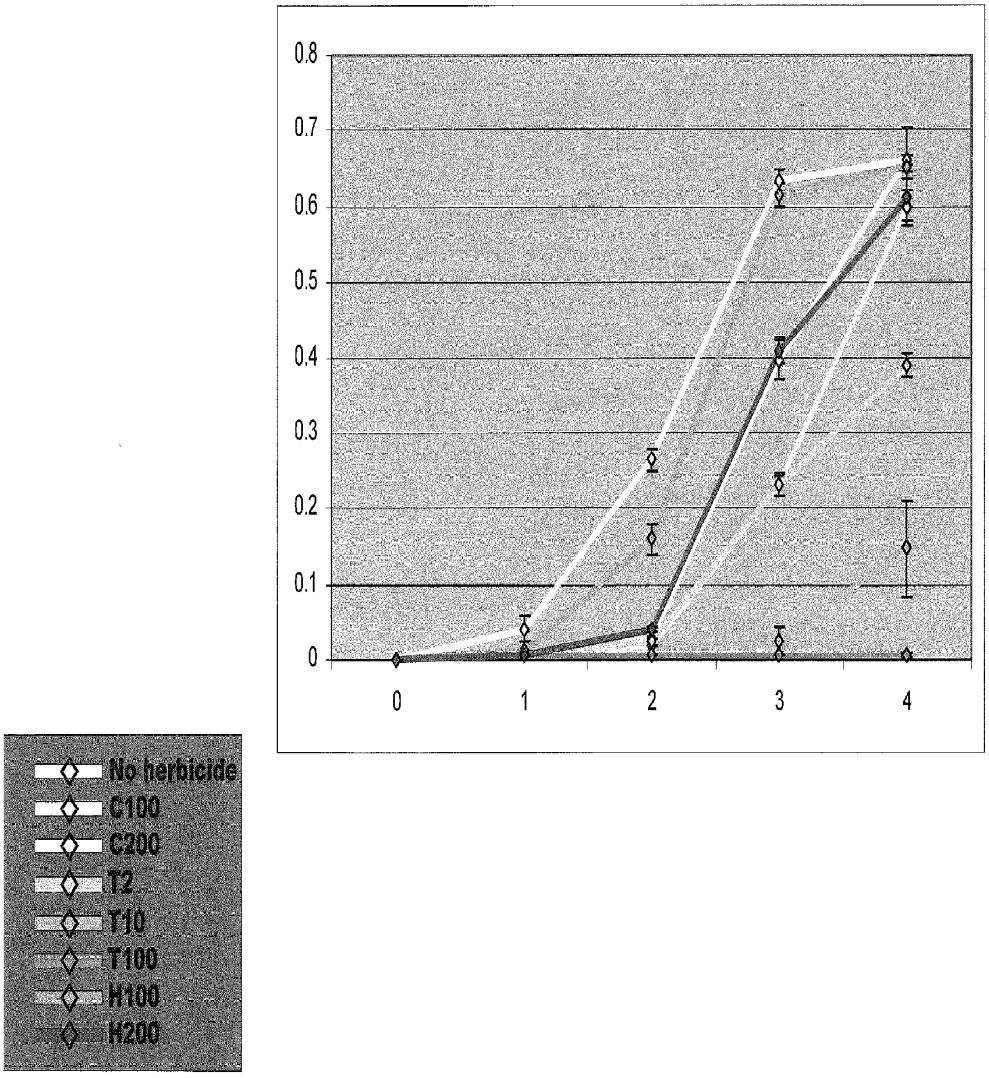


FIGURE 15

W1999G

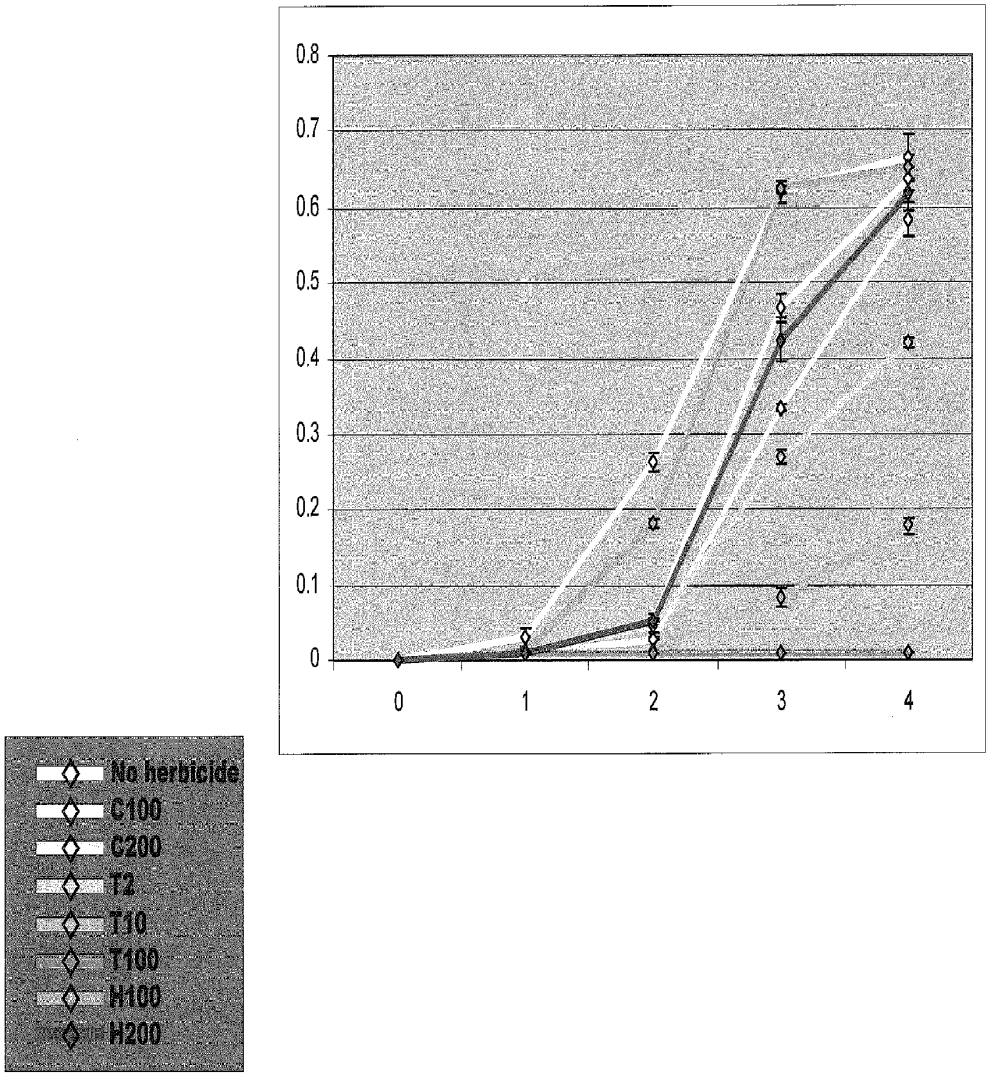


FIGURE 16

V2098A

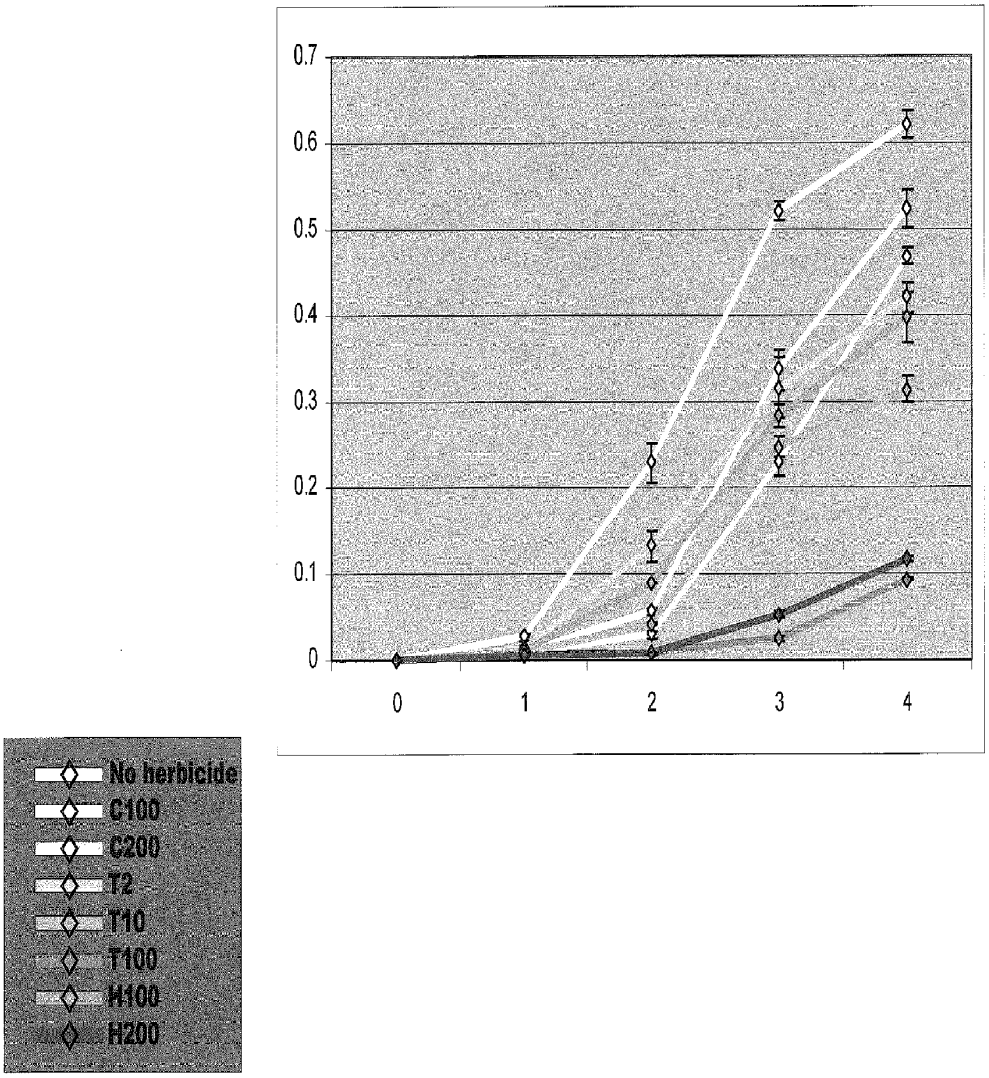


FIGURE 17

V2098A

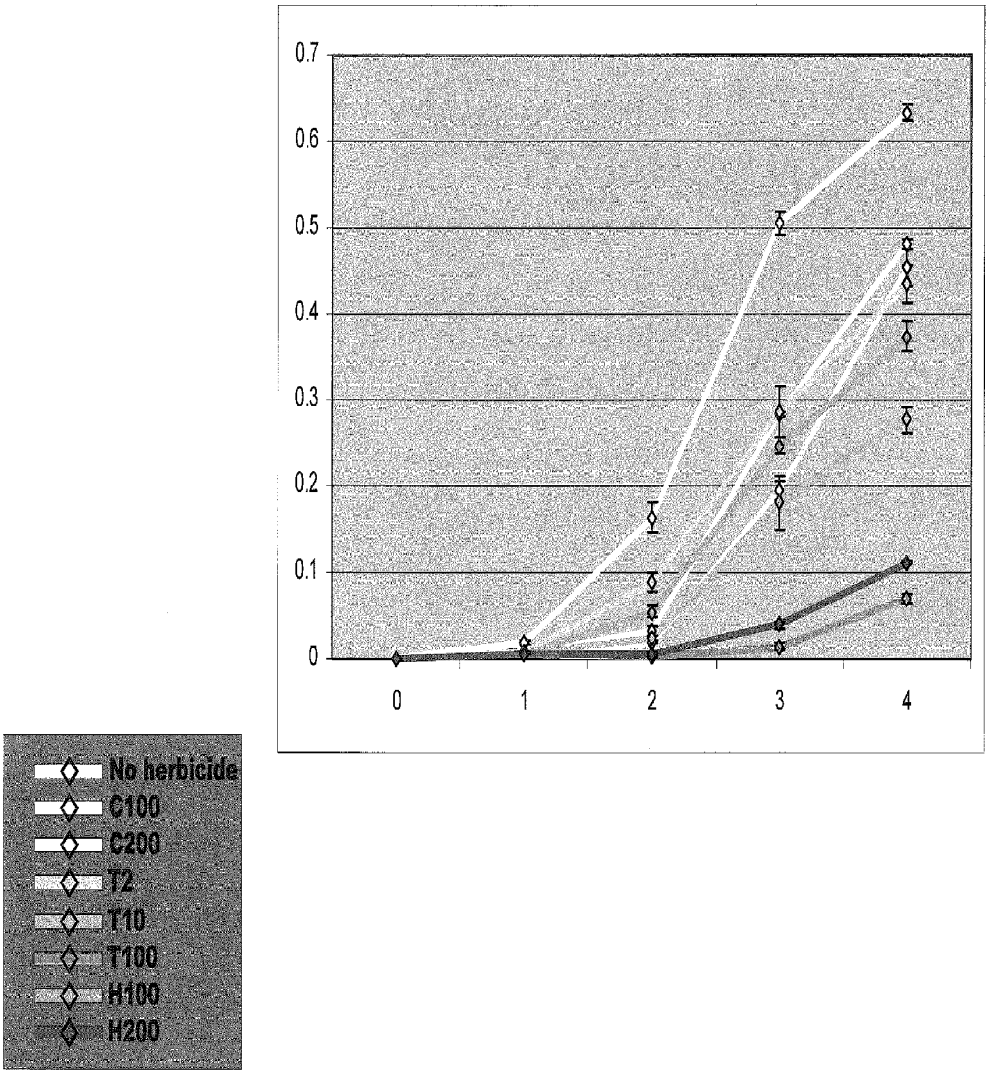


FIGURE 18

V2098A

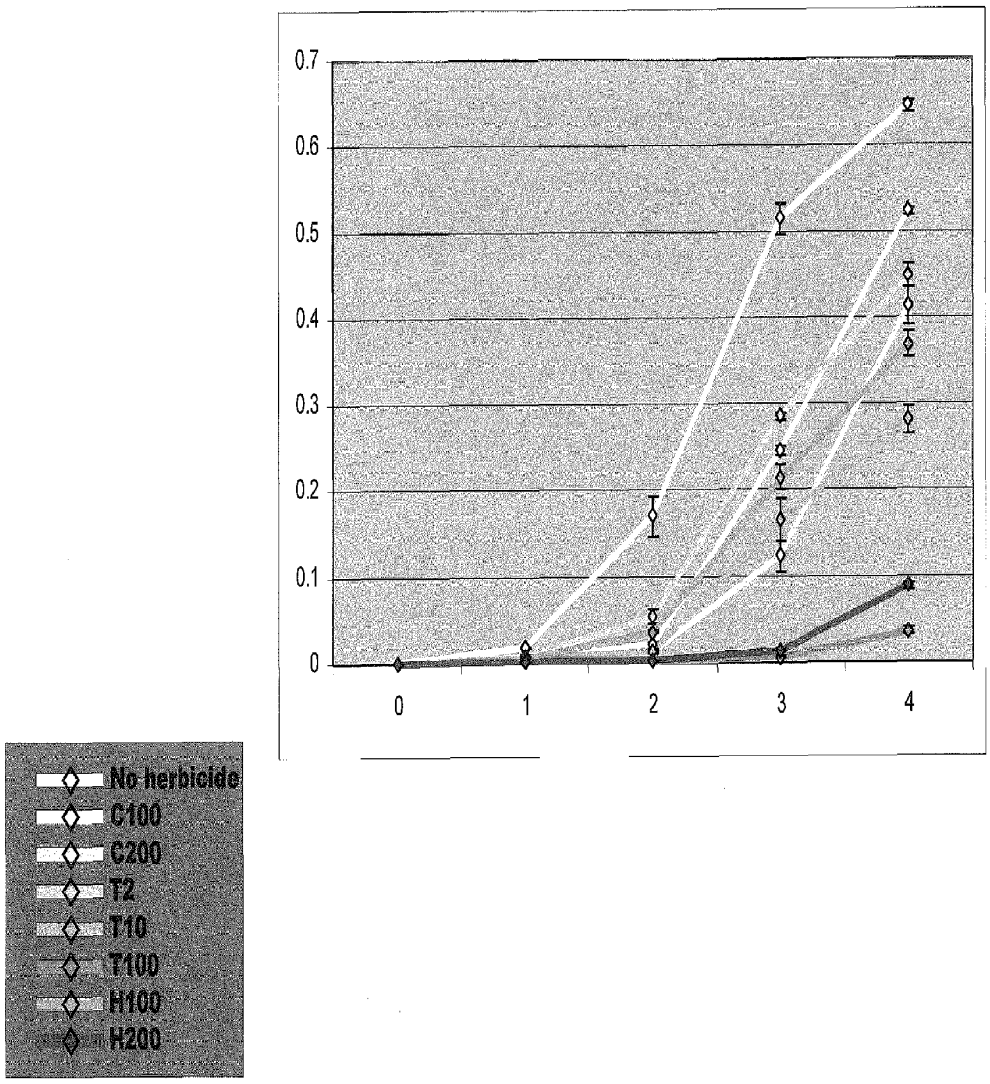


FIGURE 19

W1999G

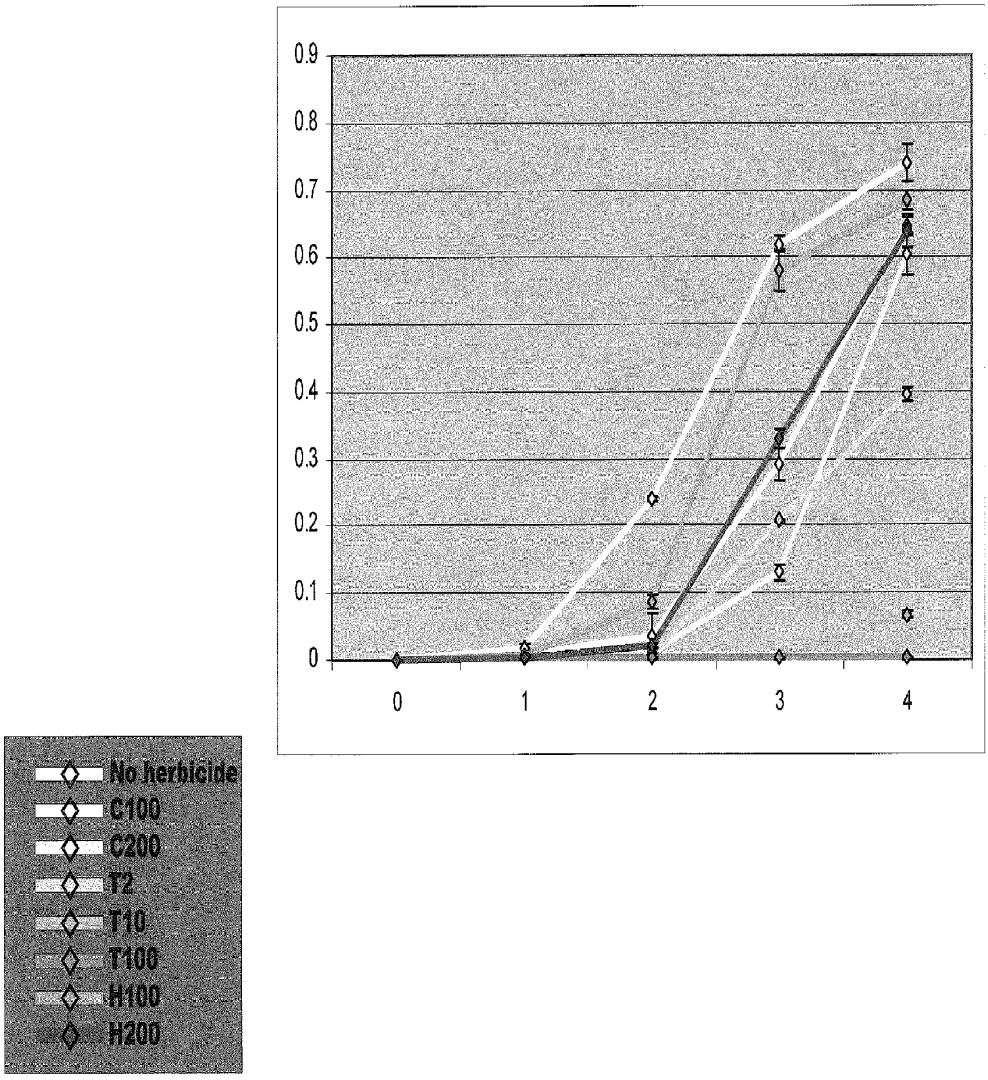


FIGURE 20

I1781L

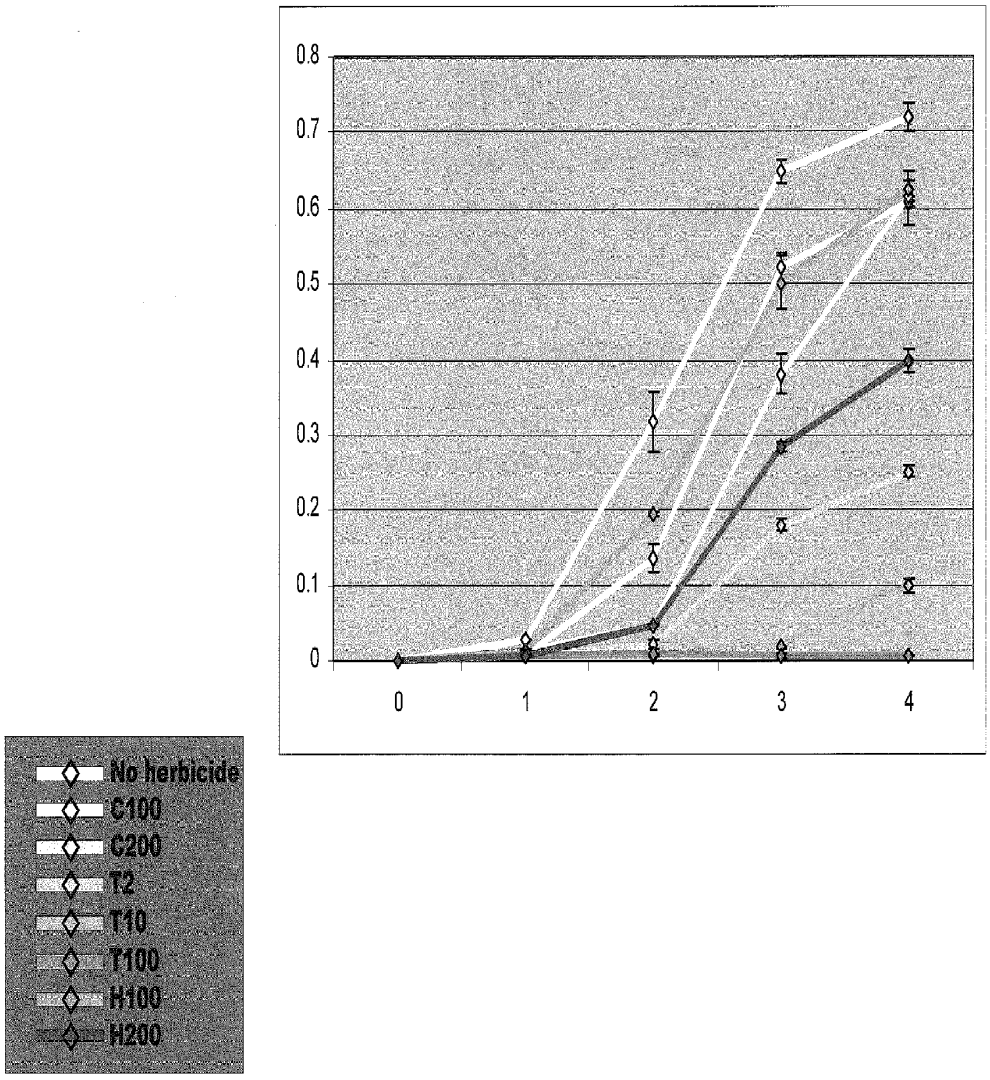


FIGURE 21

I1781L

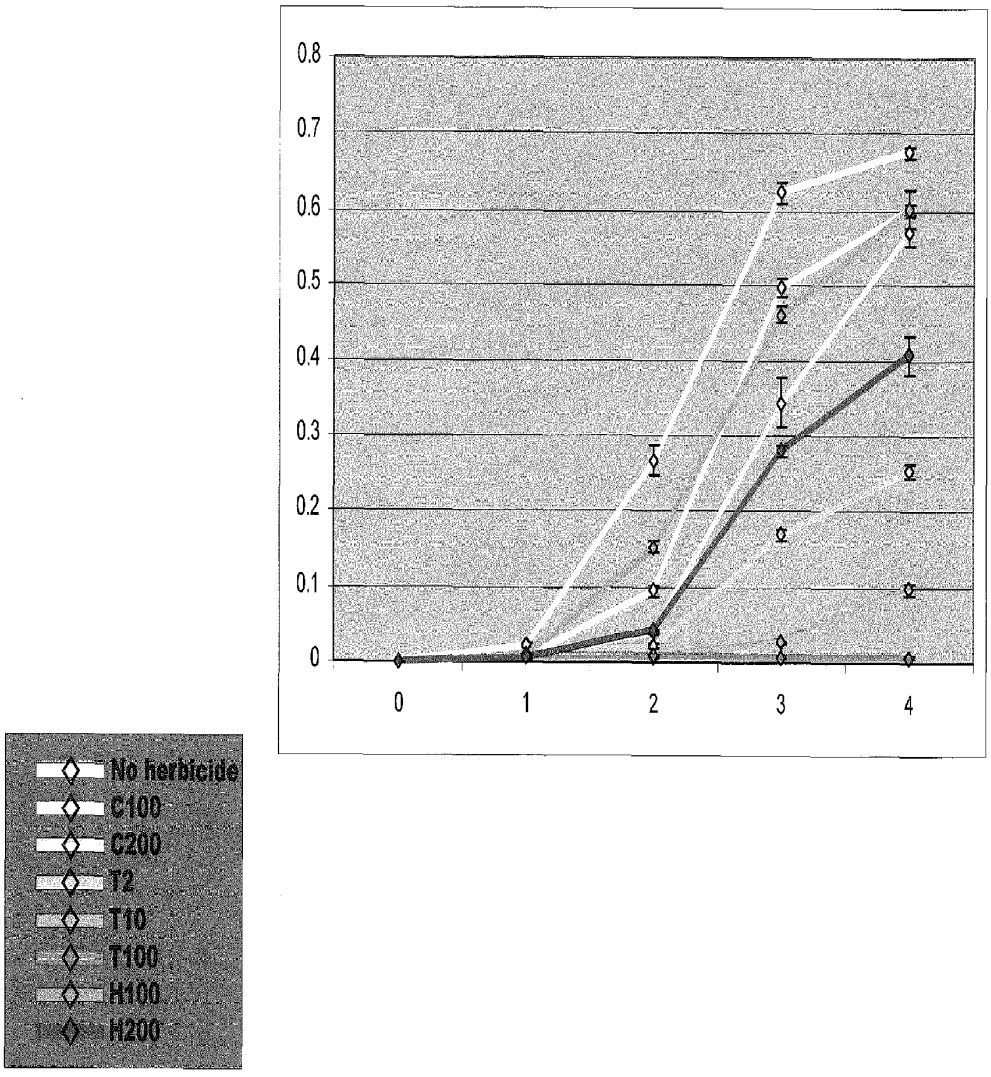


FIGURE 22

I1781L

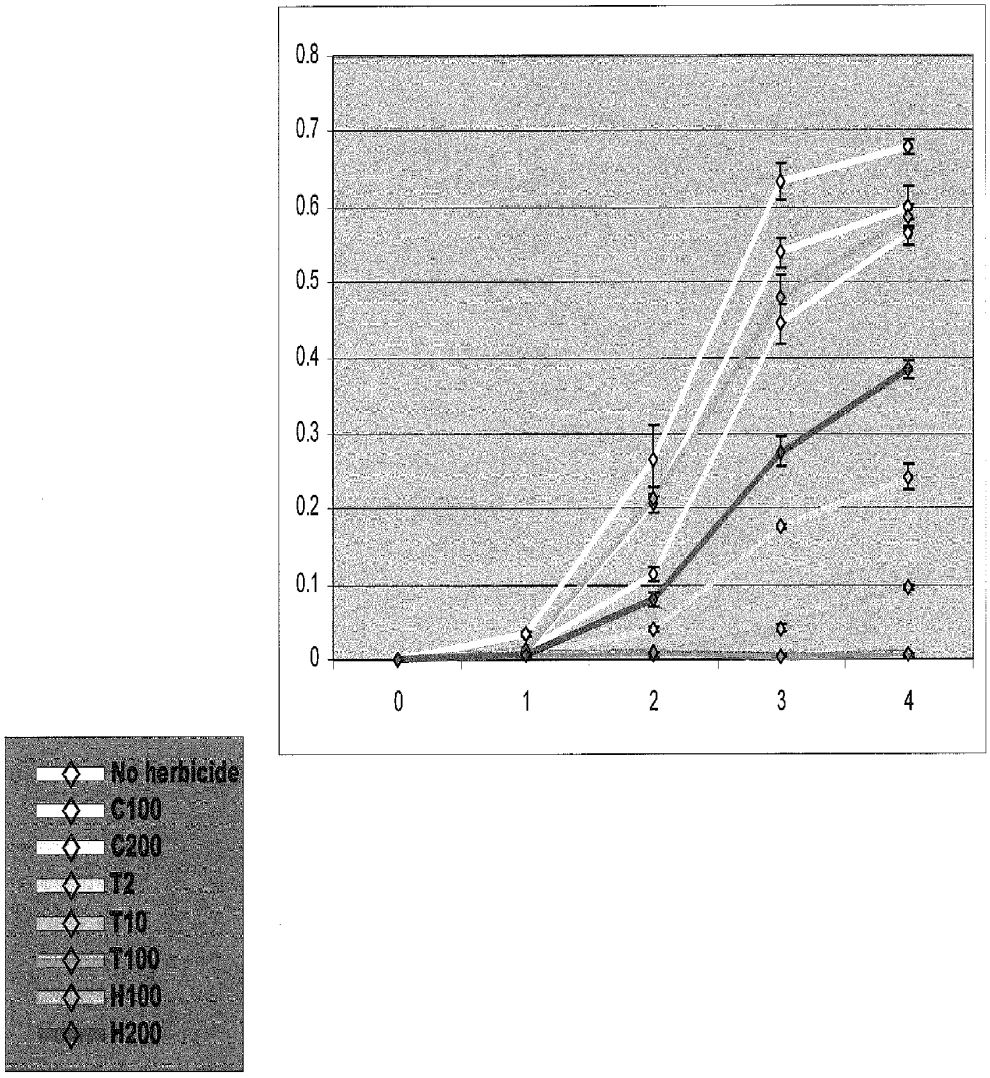


FIGURE 23

V2075L

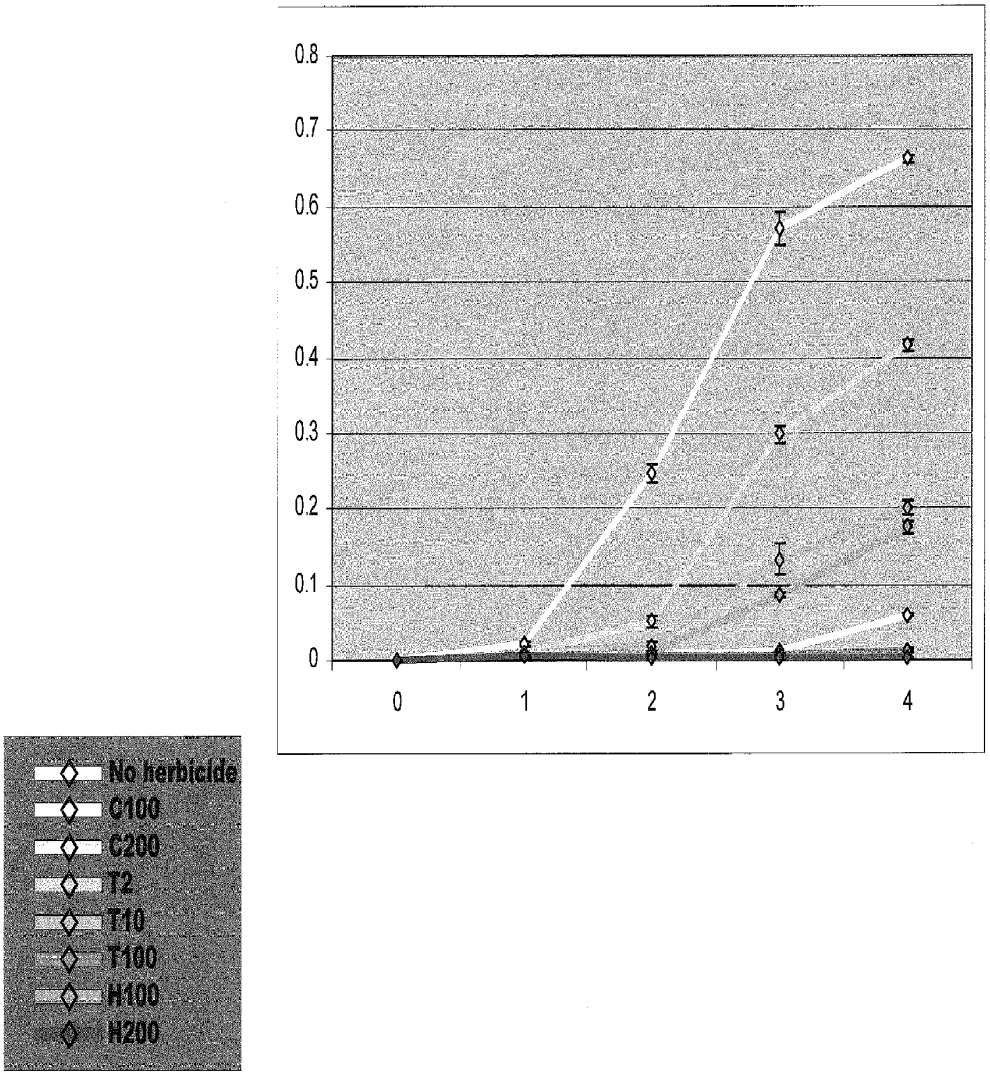


FIGURE 24

D2078G

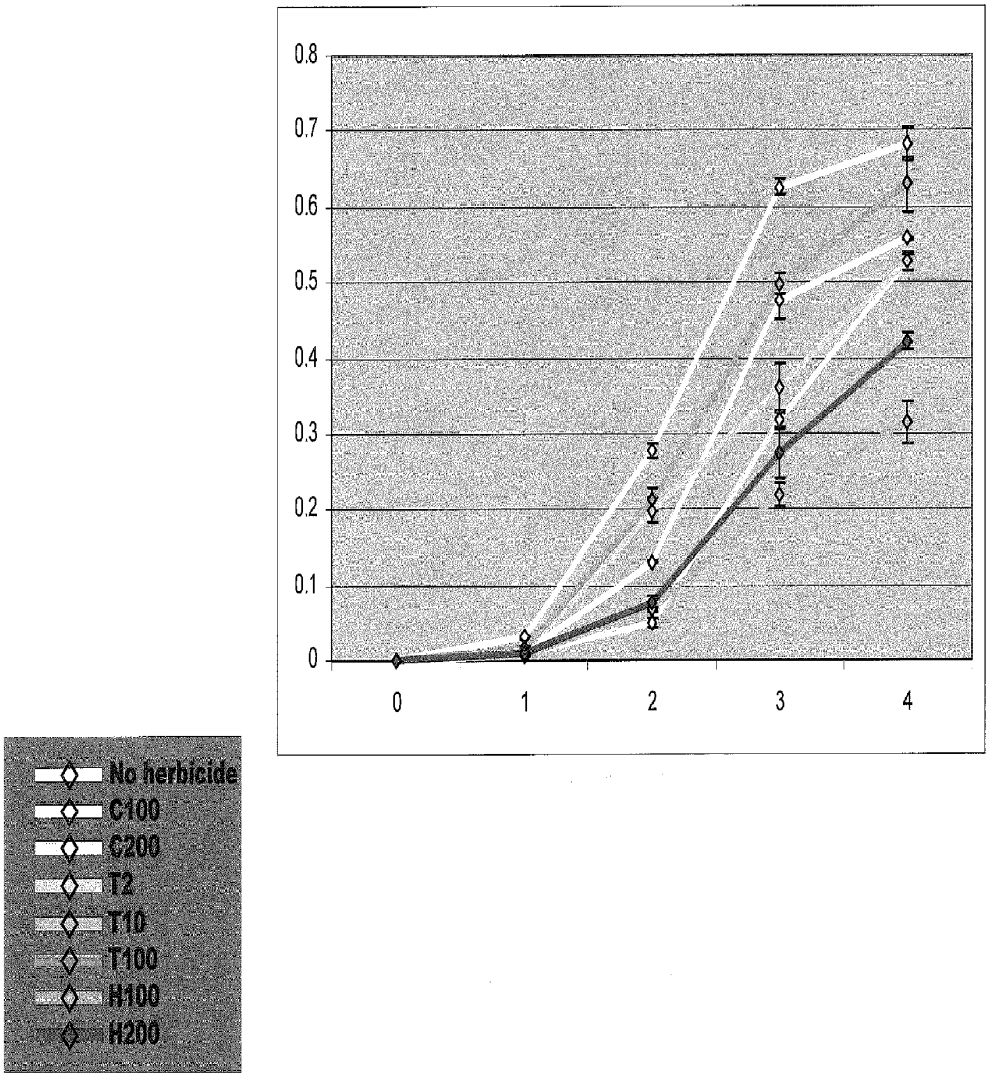


FIGURE 25

C2088R

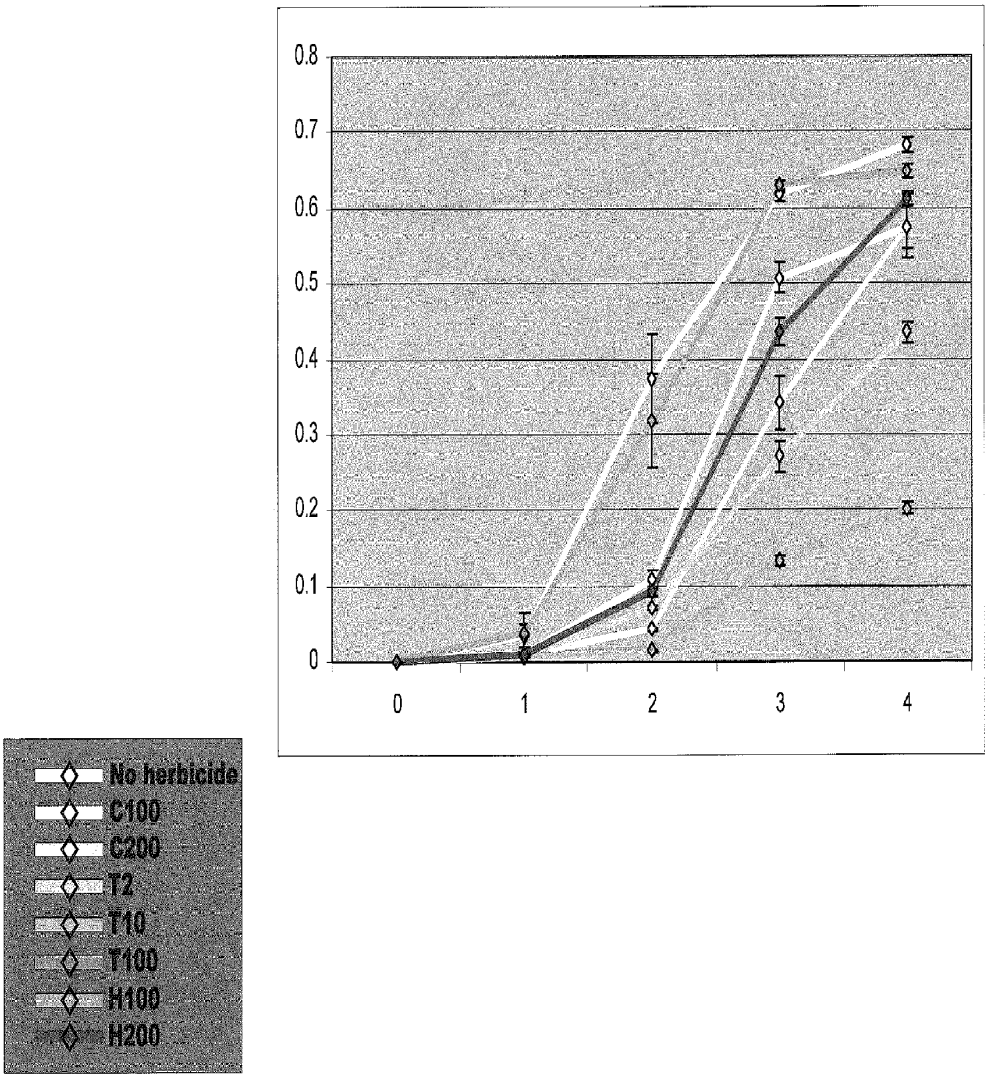


FIGURE 26

G2096A

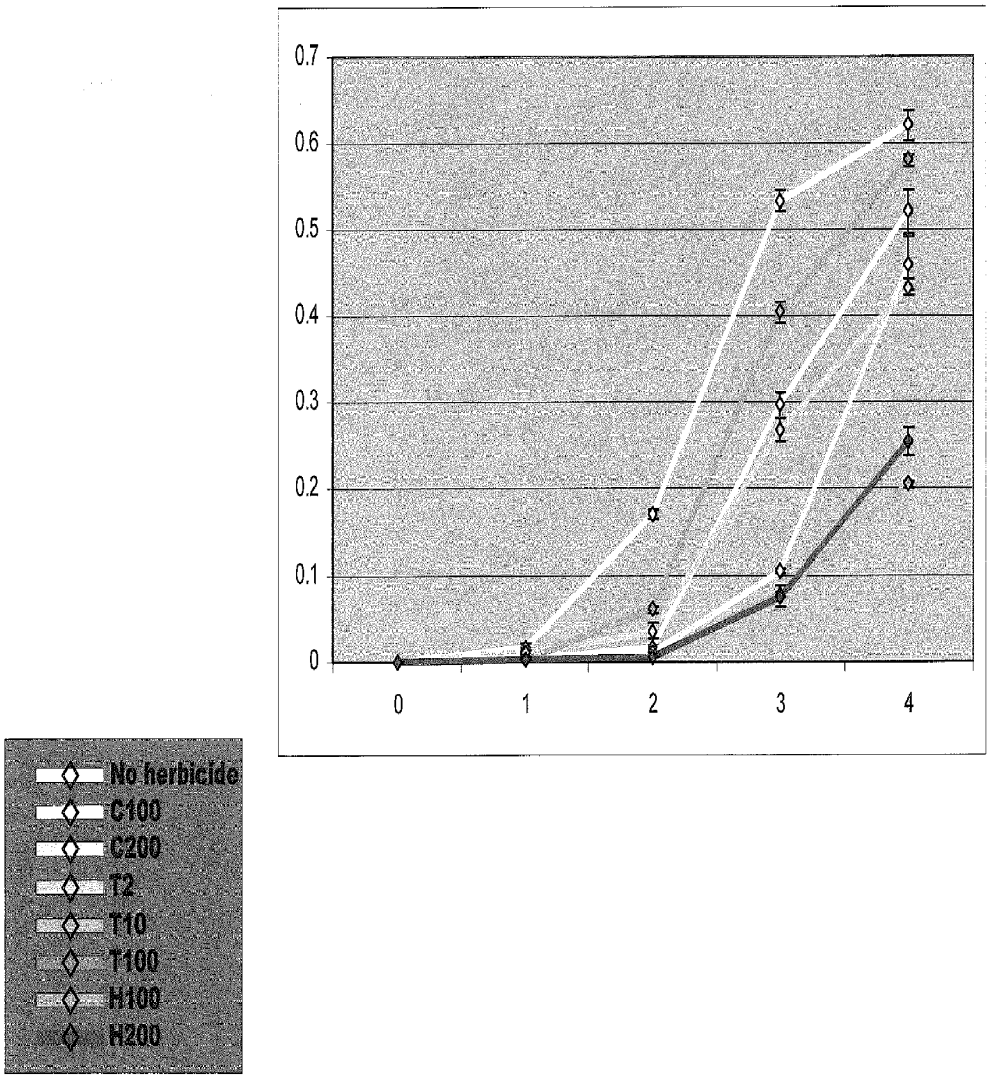


FIGURE 27

G2096S

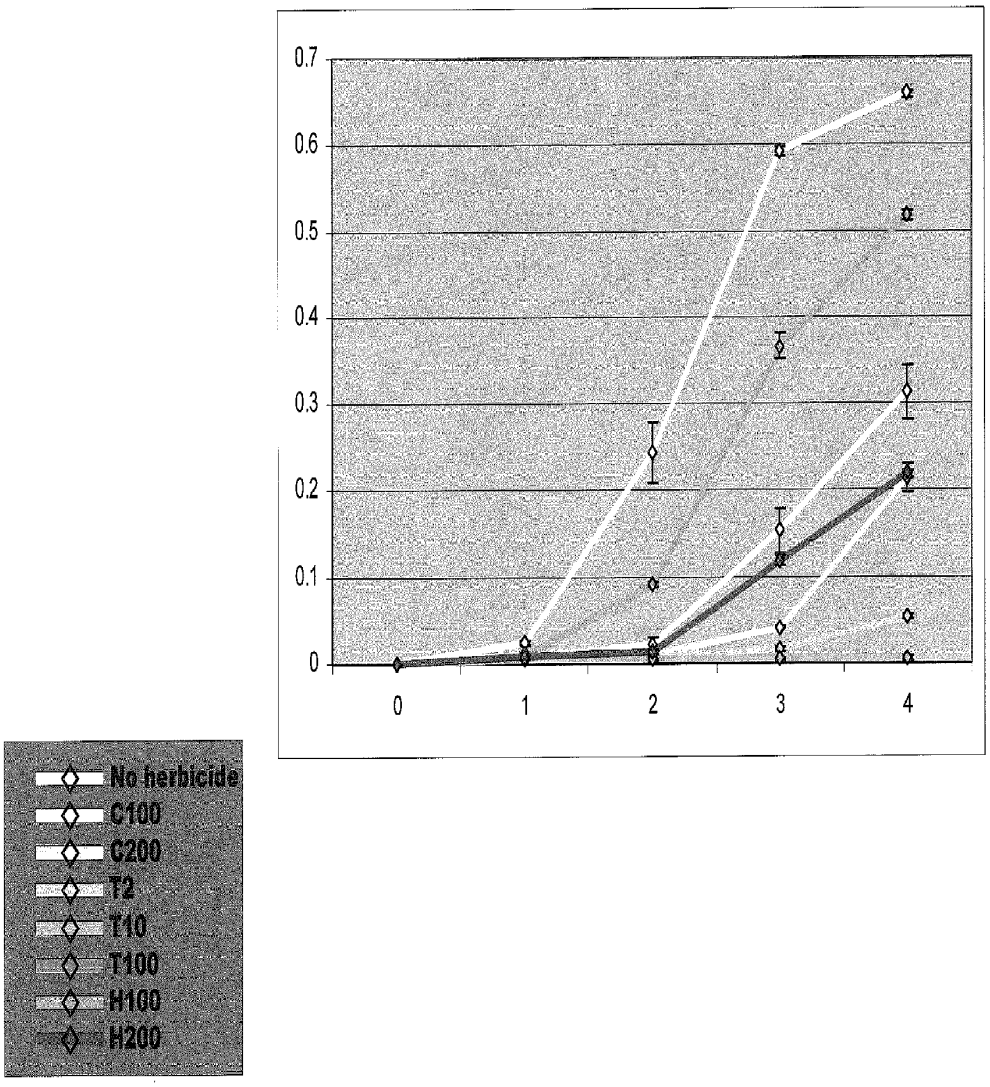


FIGURE 28

I1811N

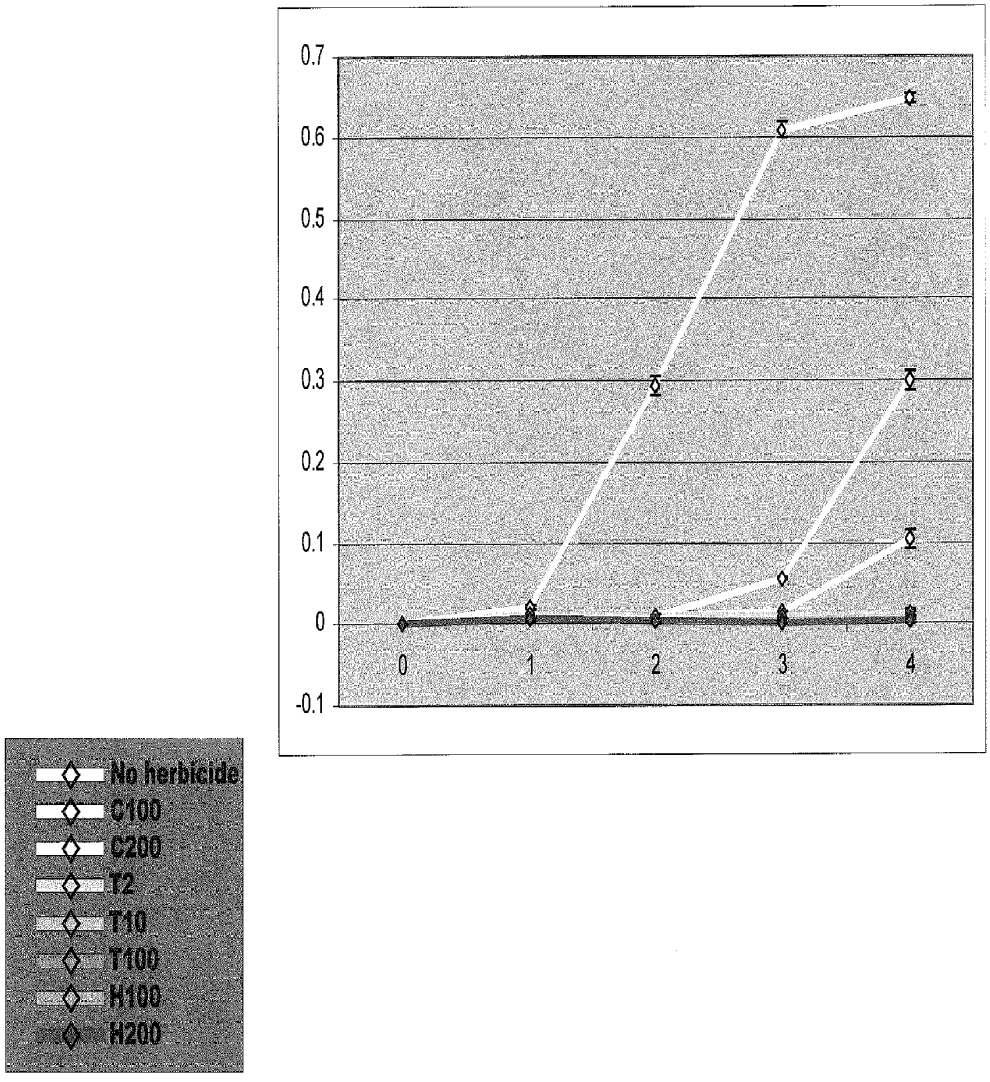


FIGURE 29

A1786P

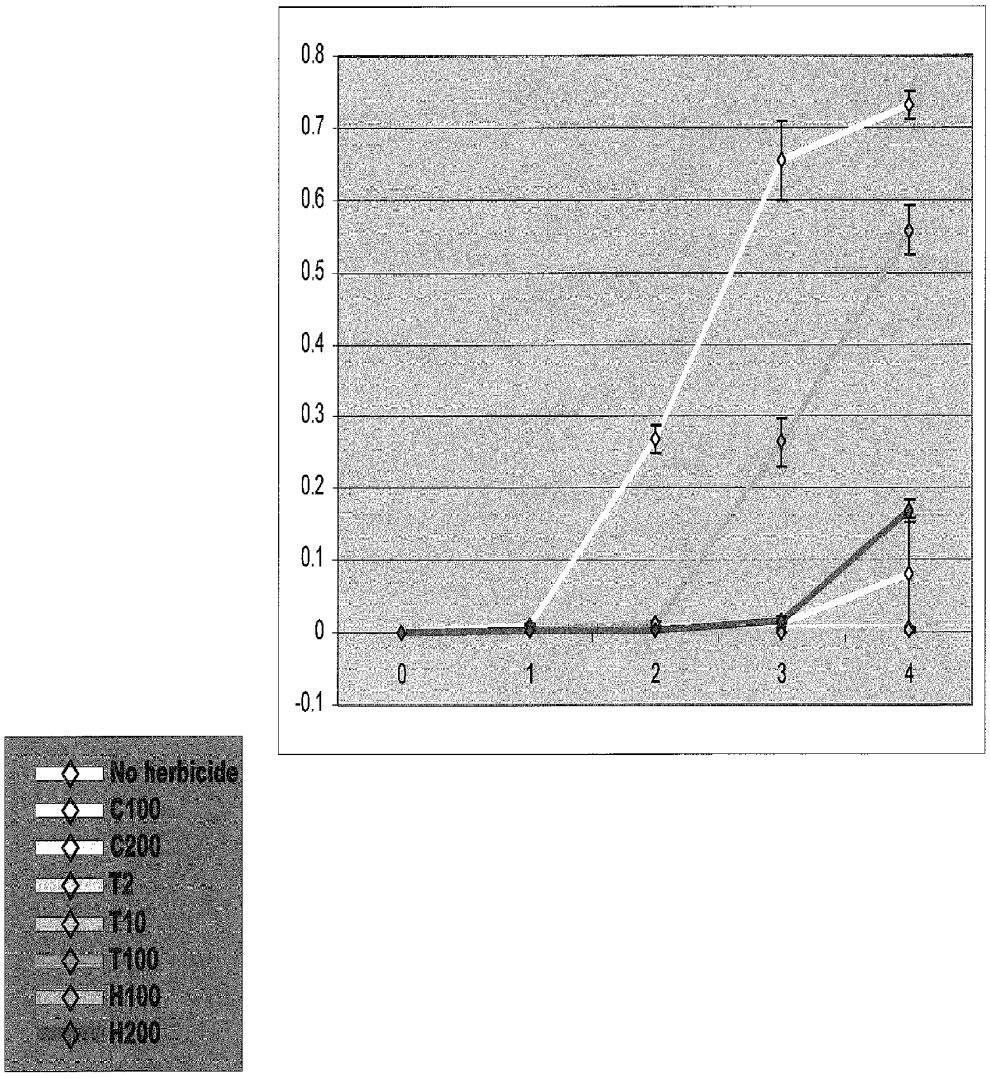


FIGURE 30

I1781V

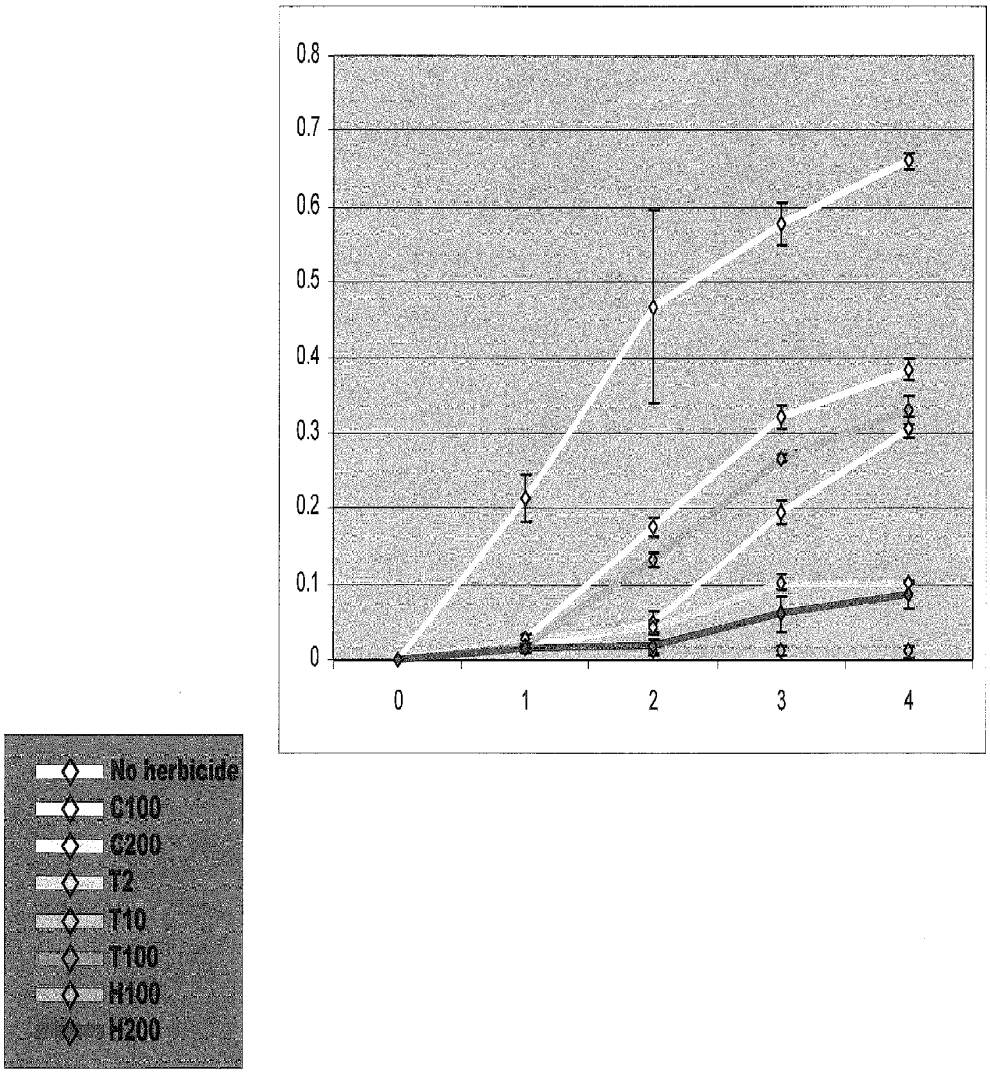


FIGURE 31

D2078T

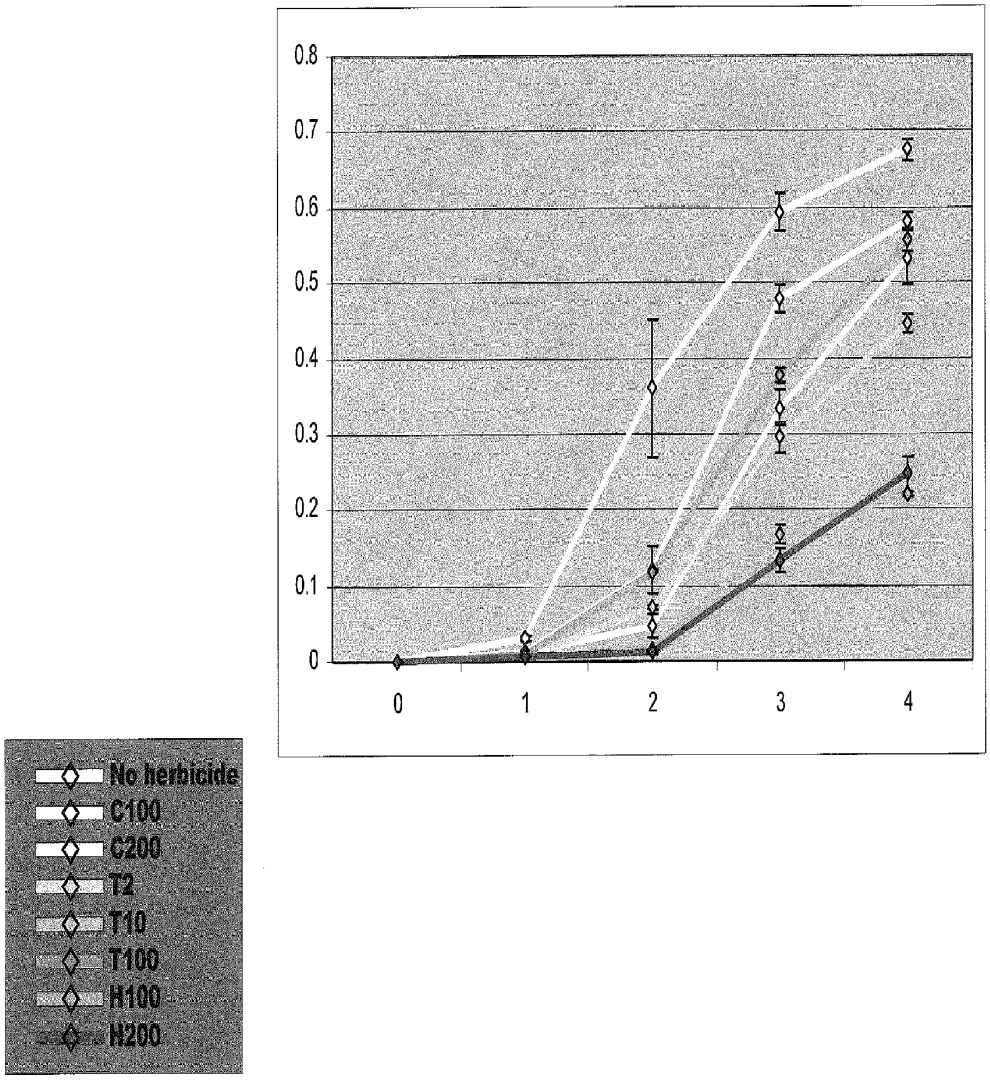


FIGURE 32

V2098H

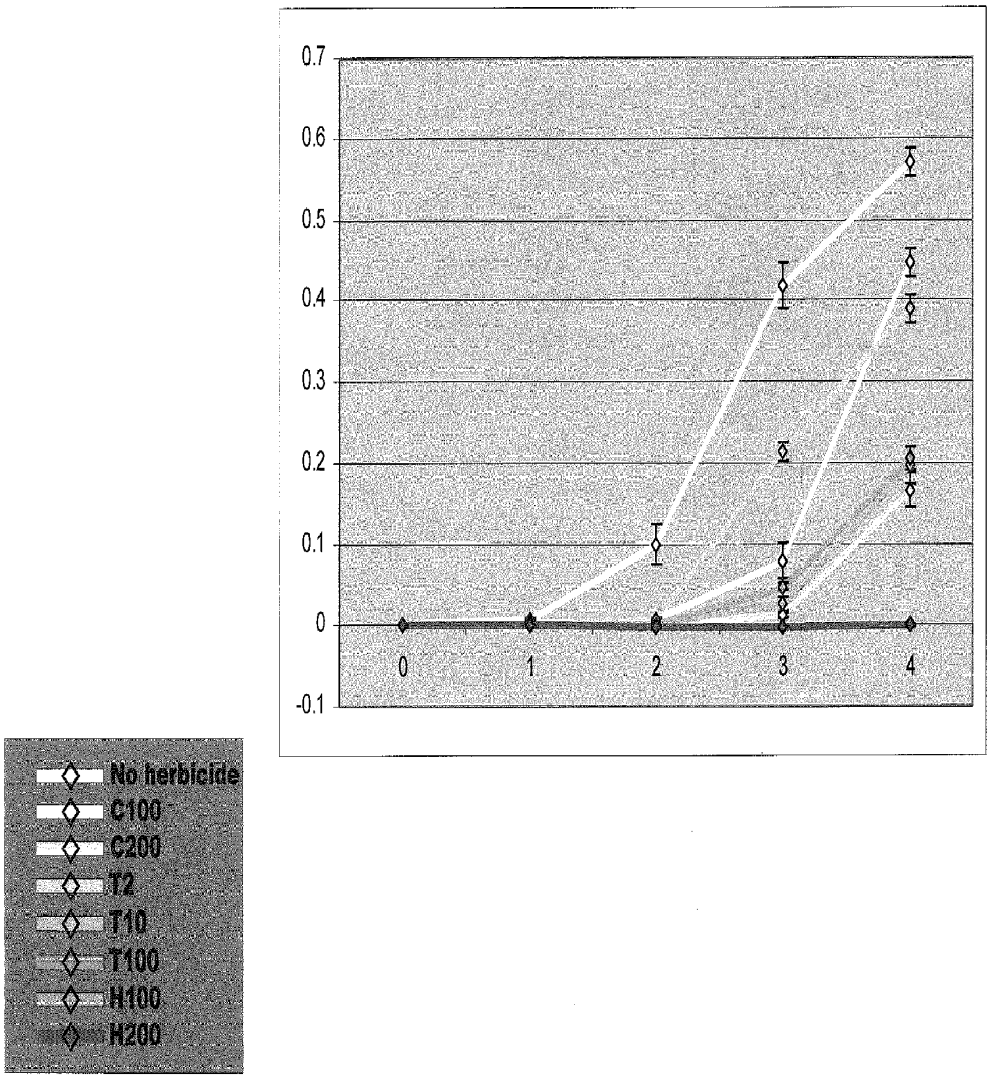


FIGURE 33

W2074L

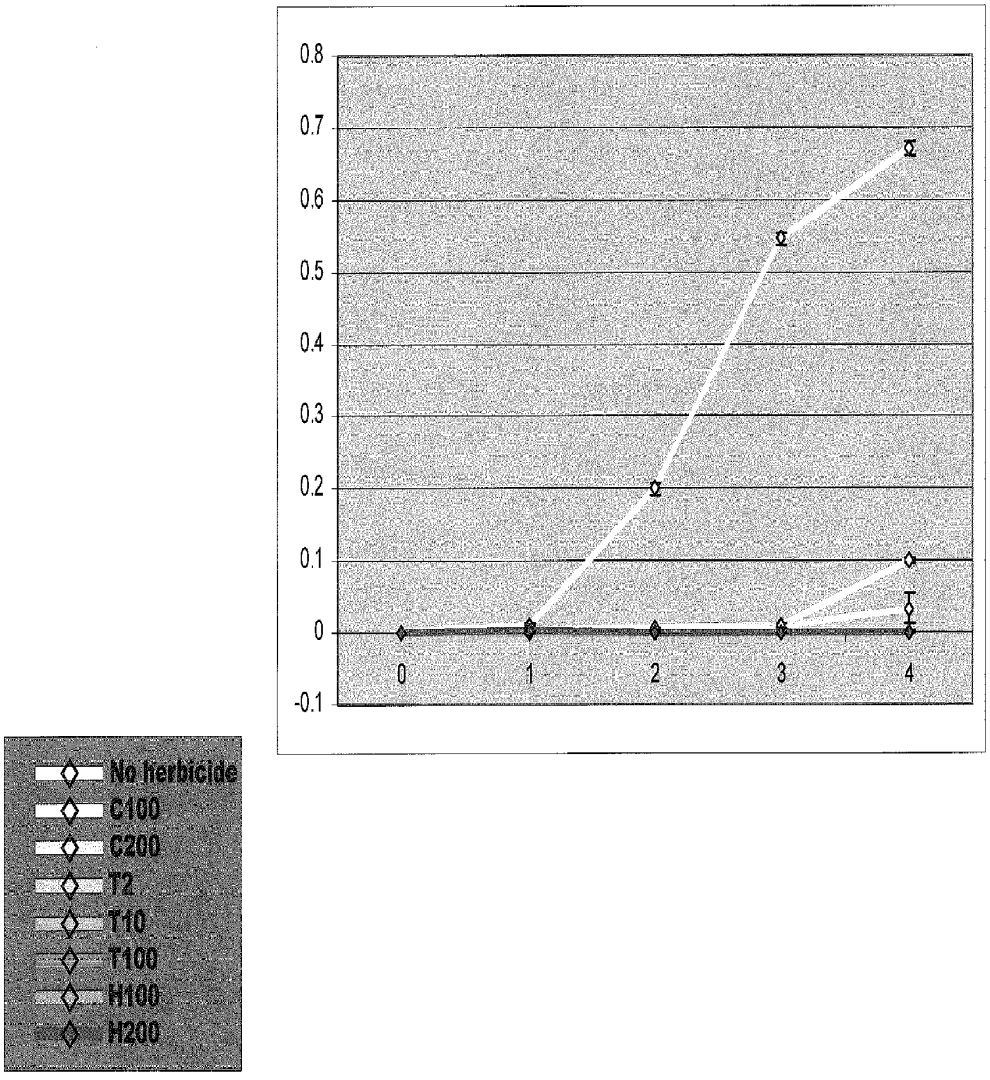


FIGURE 34

I1781T

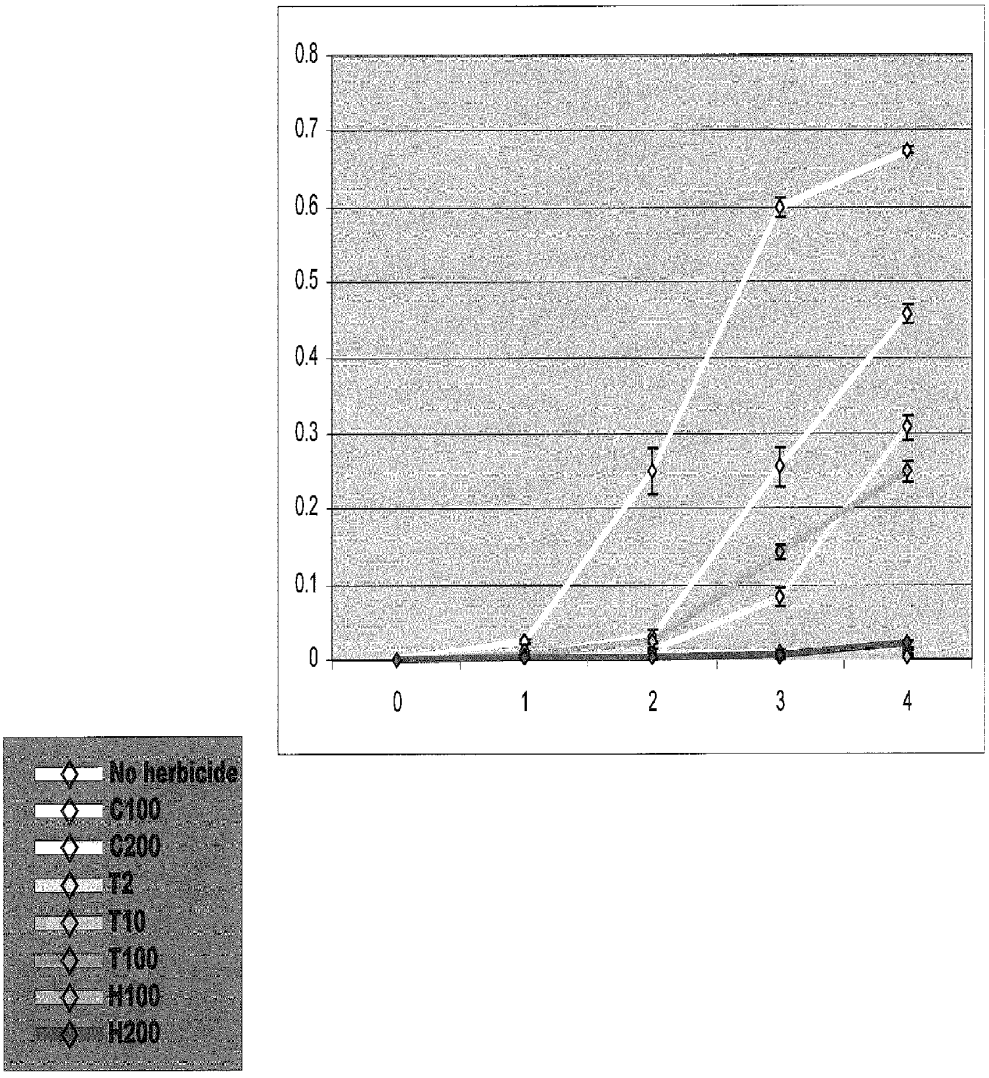


FIGURE 35

C2088W

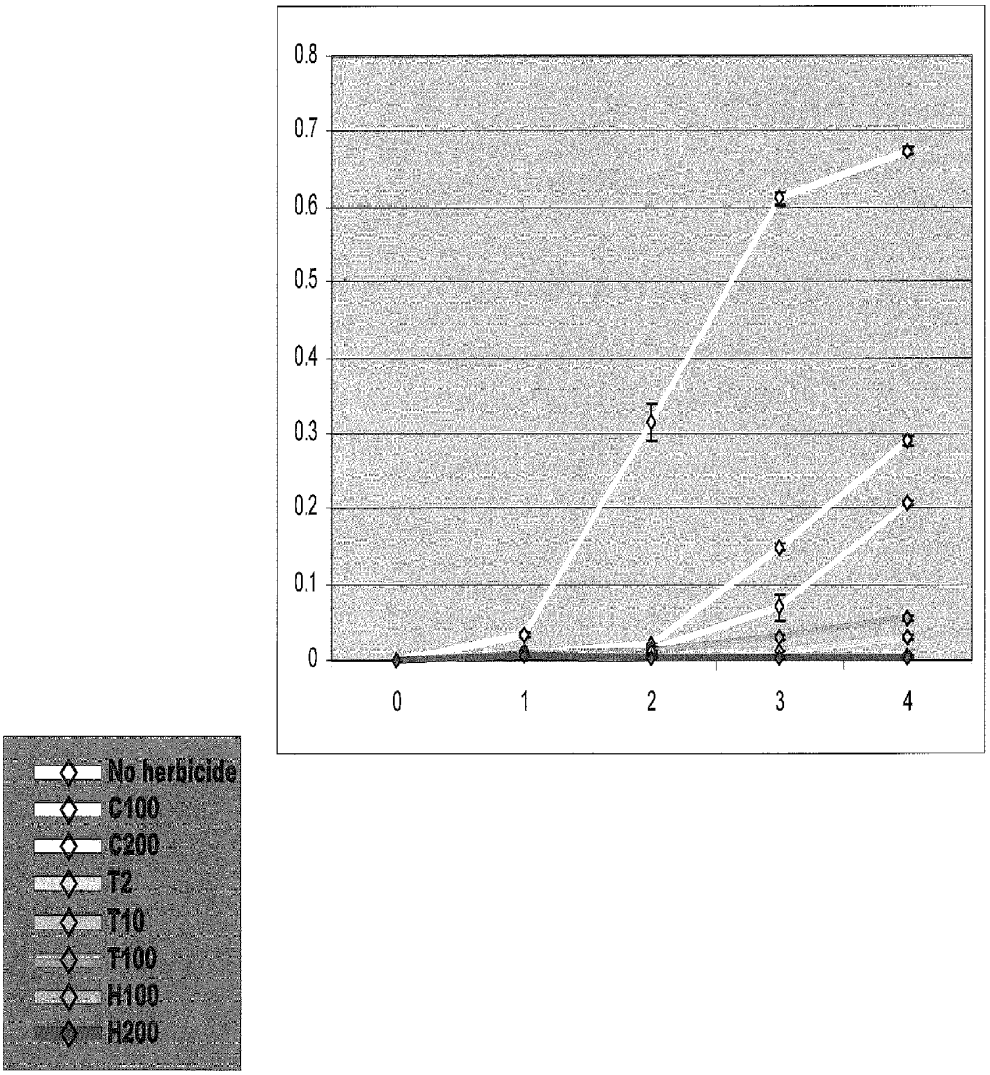


FIGURE 36

ΔK2080ΔI2081

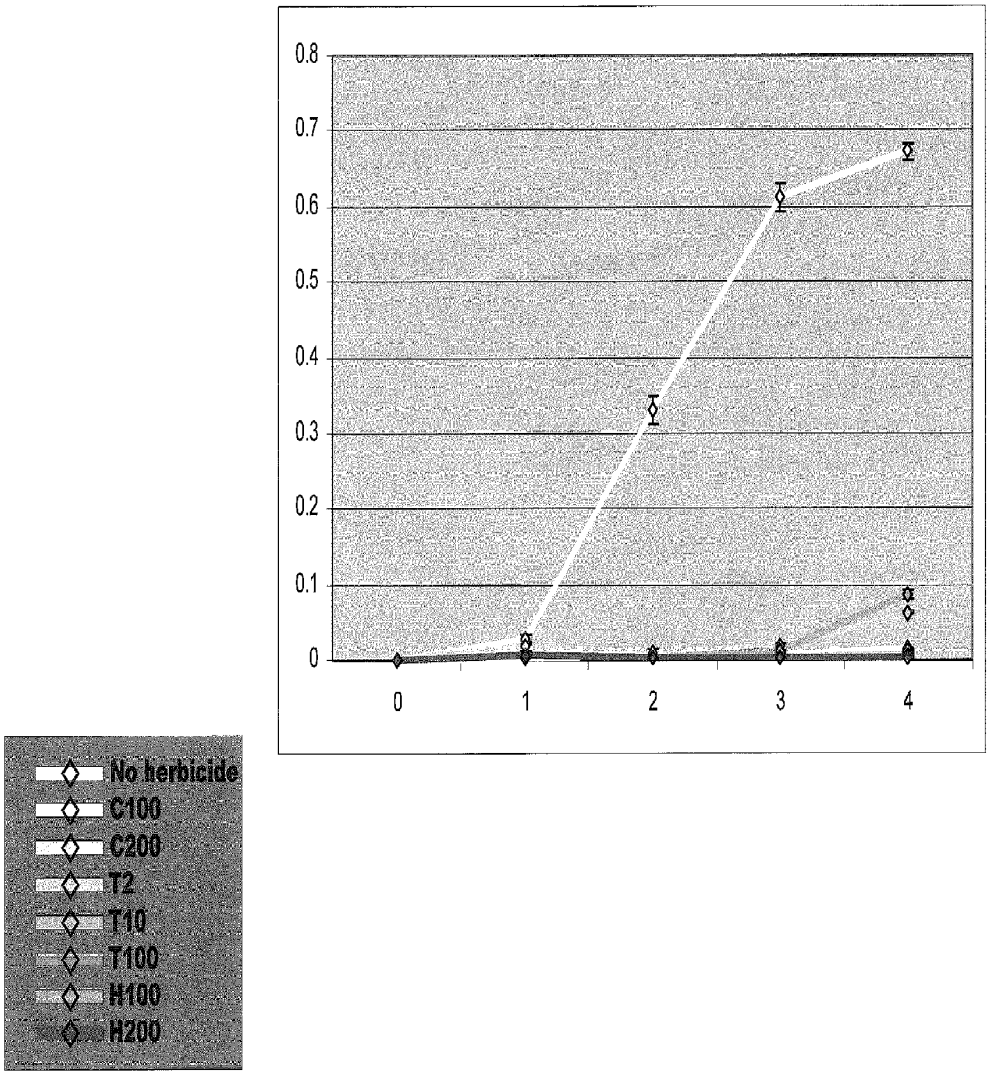


FIGURE 37

A1785G

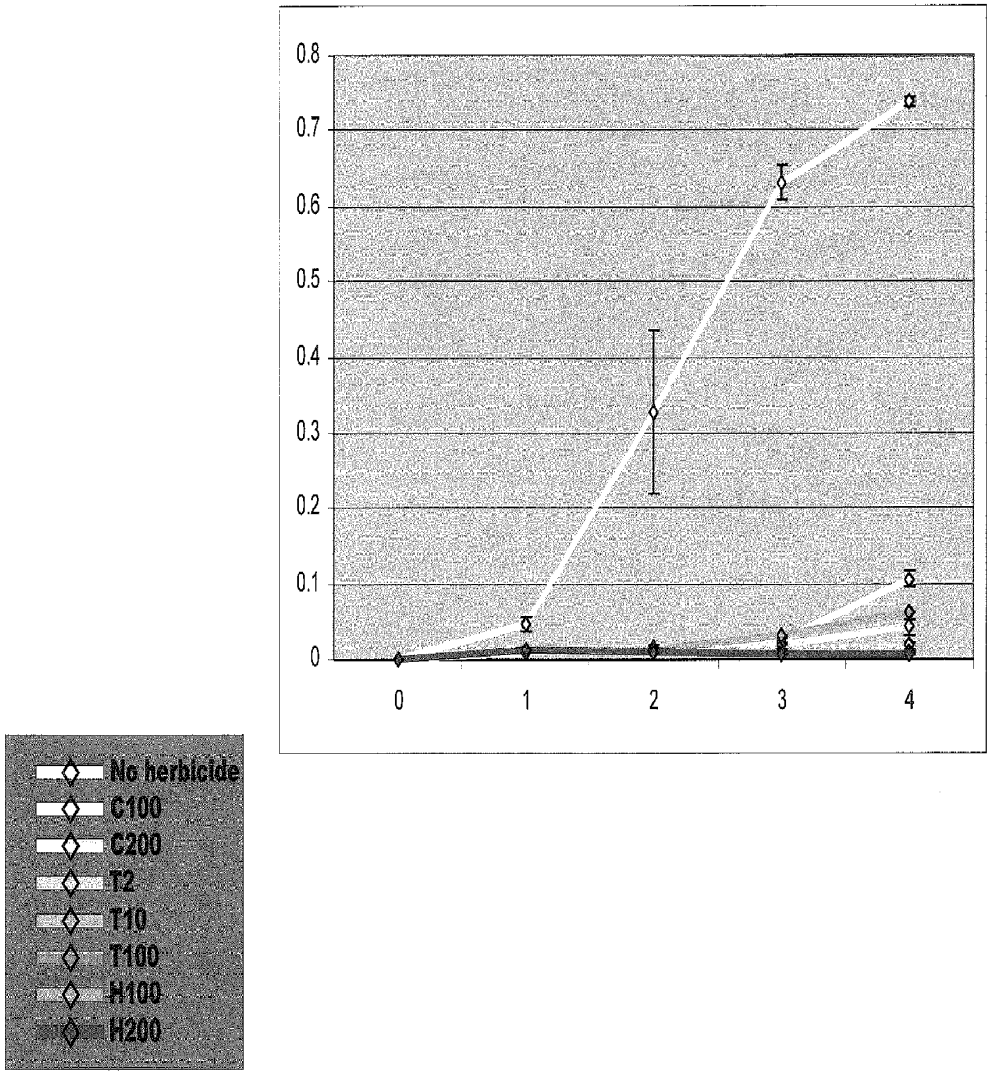


FIGURE 38

V2075I

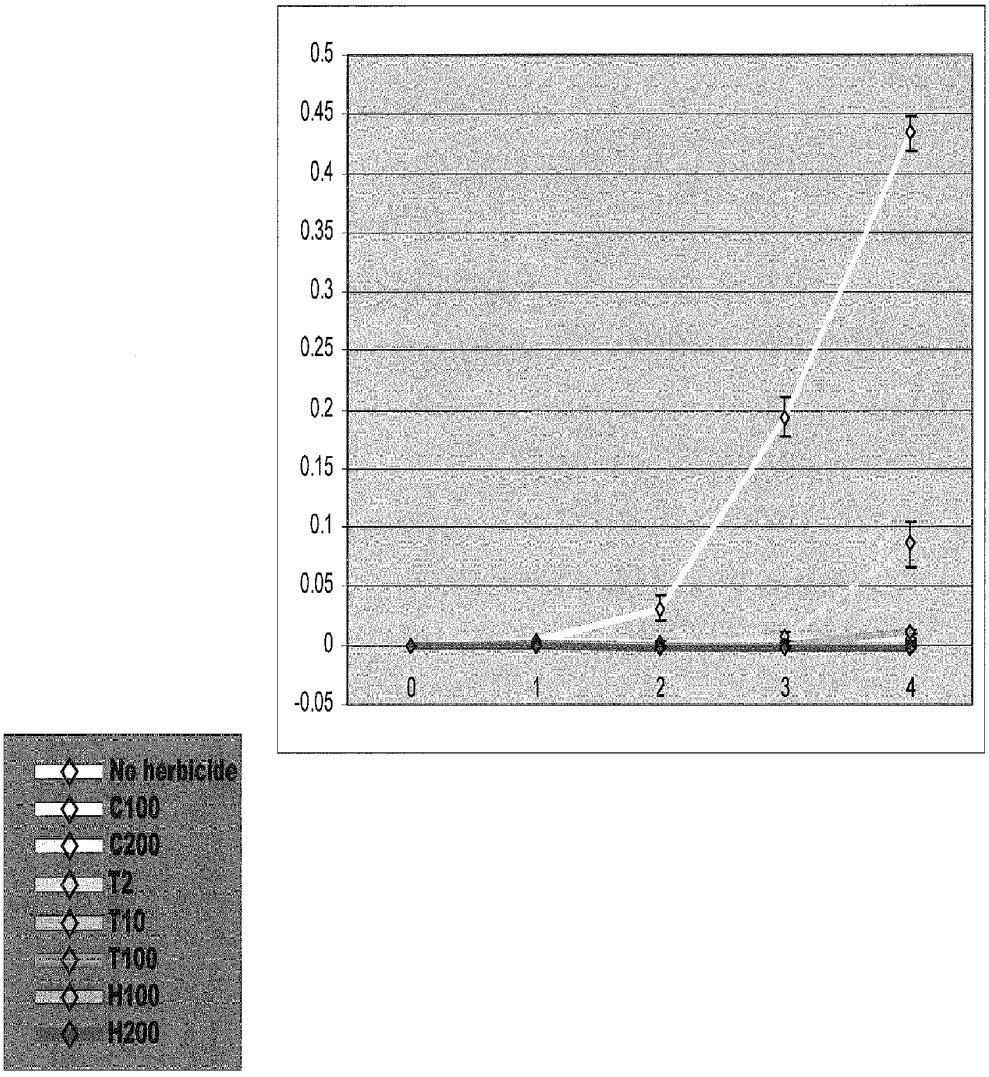


FIGURE 39

V2075M

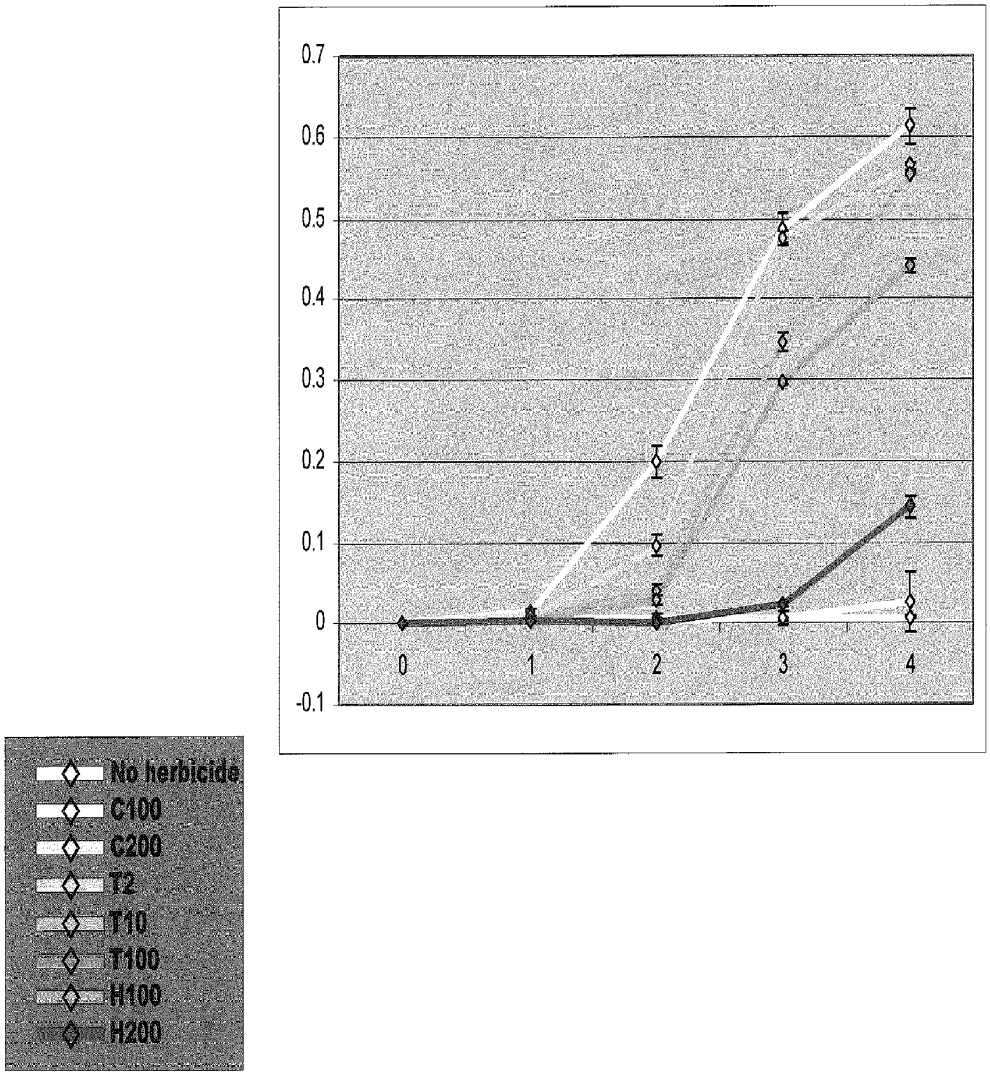


FIGURE 40

V2098S

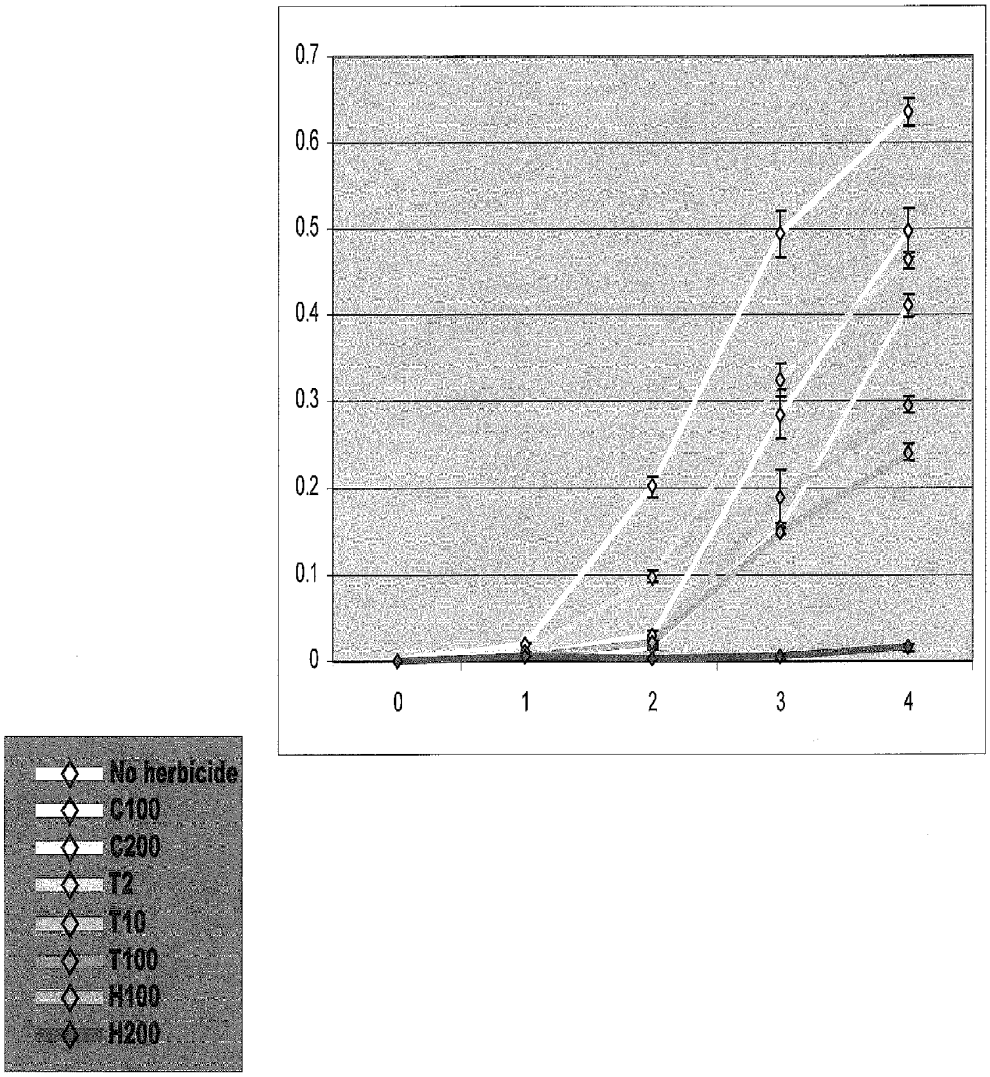


FIGURE 41

V2098G

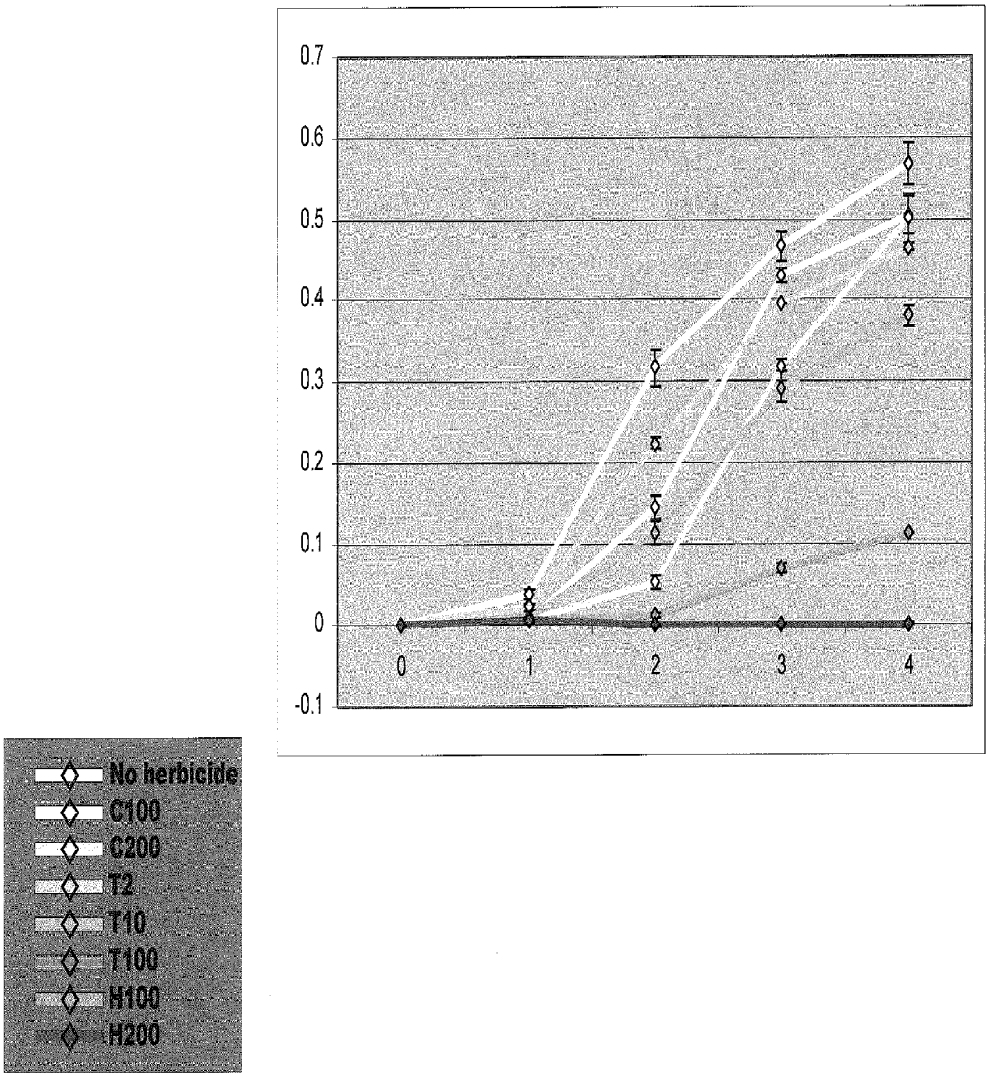


FIGURE 42

V2098P

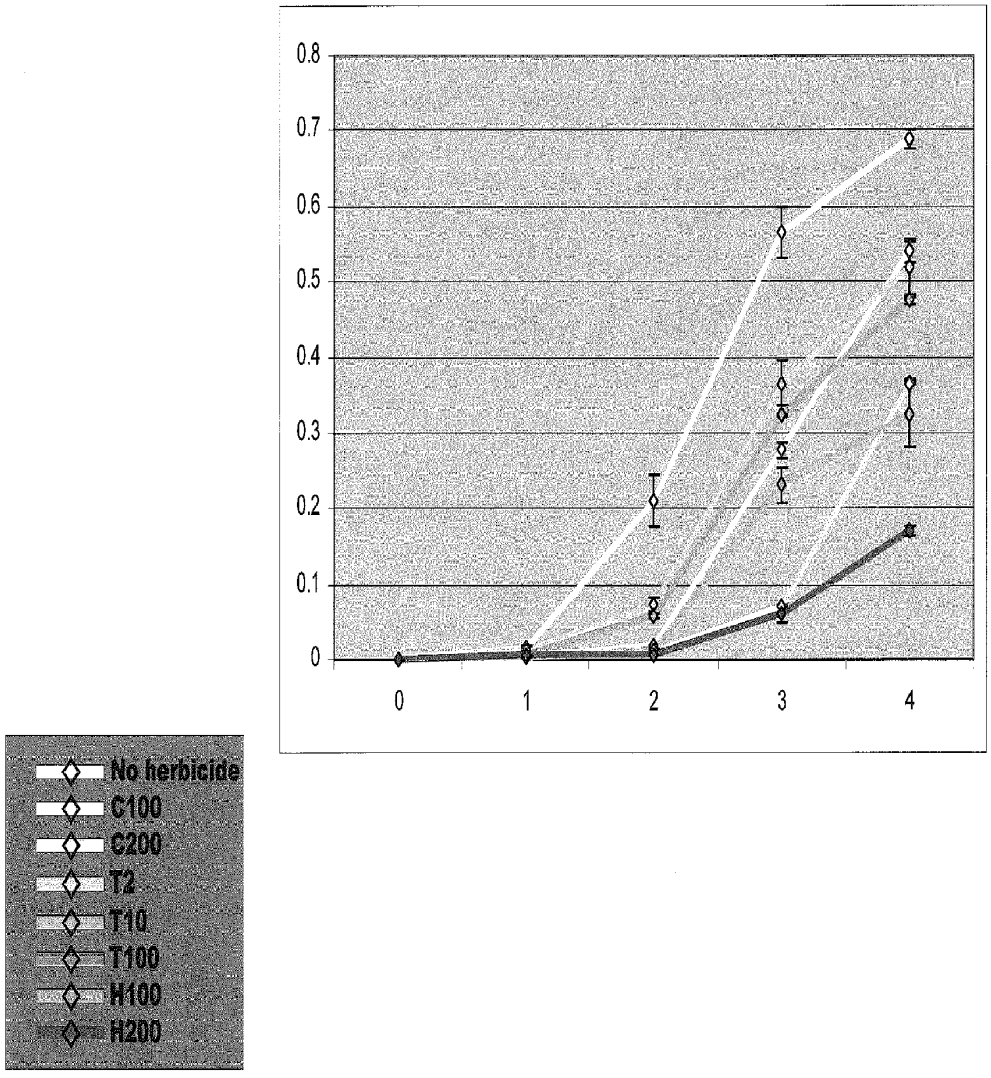


FIGURE 43

V2049F

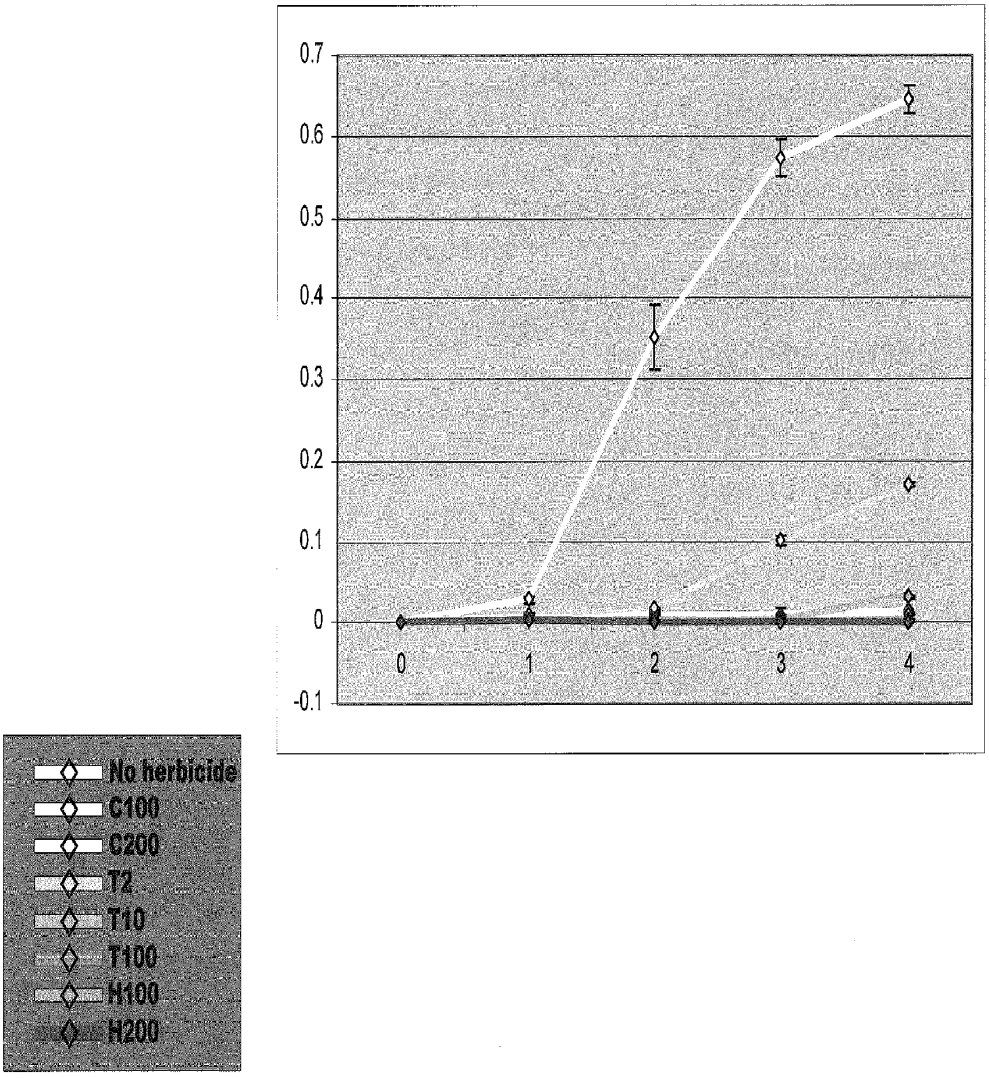


FIGURE 44

G1783C

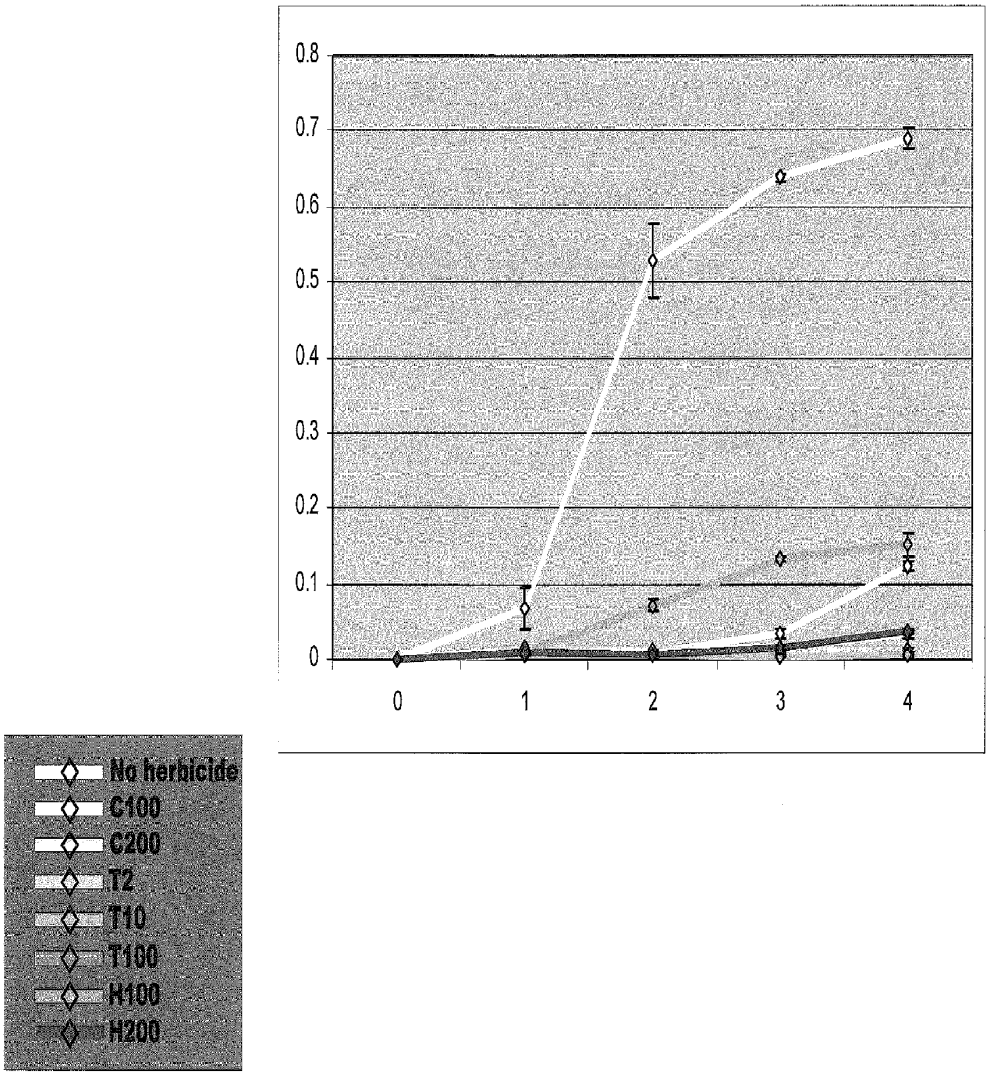


FIGURE 45

V2075G

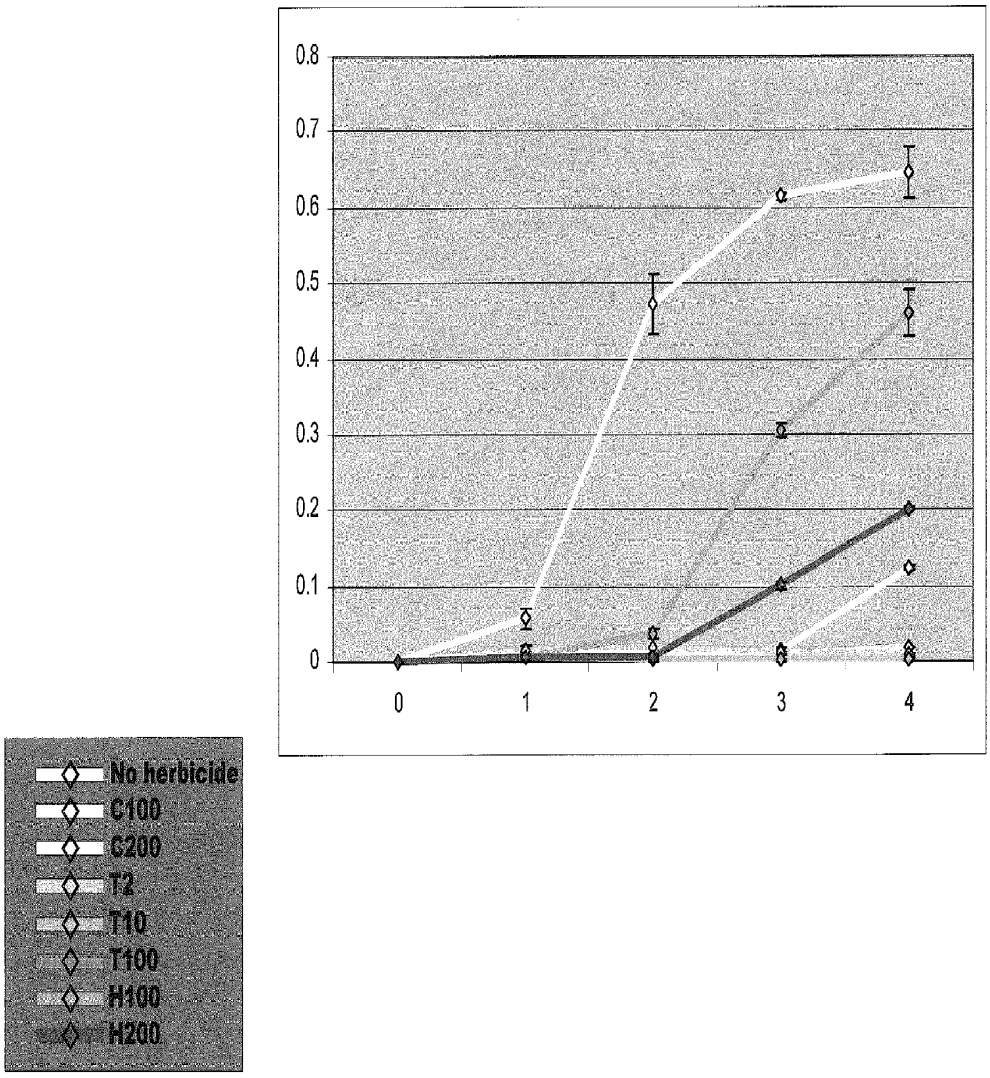


FIGURE 46

Q1824P D2078G

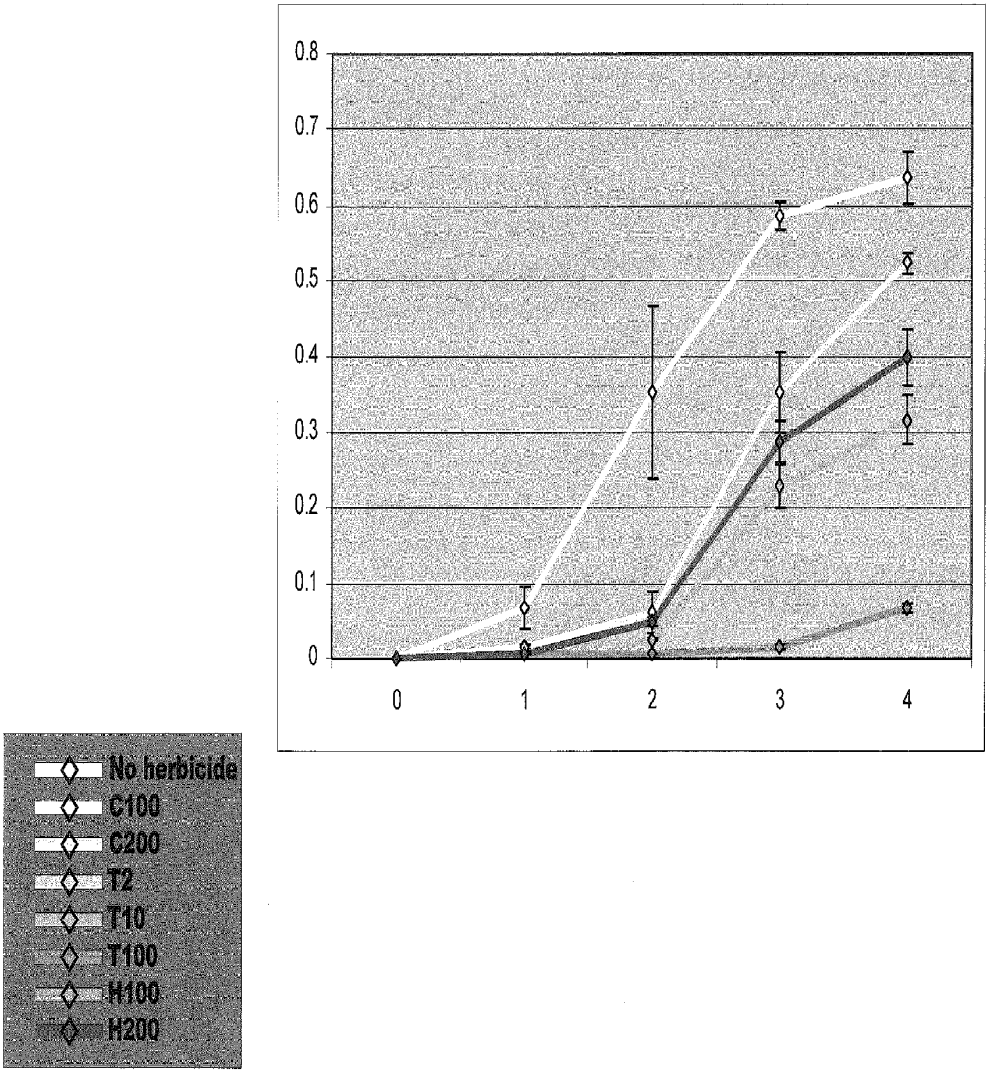


FIGURE 47

I1781L Q1824P

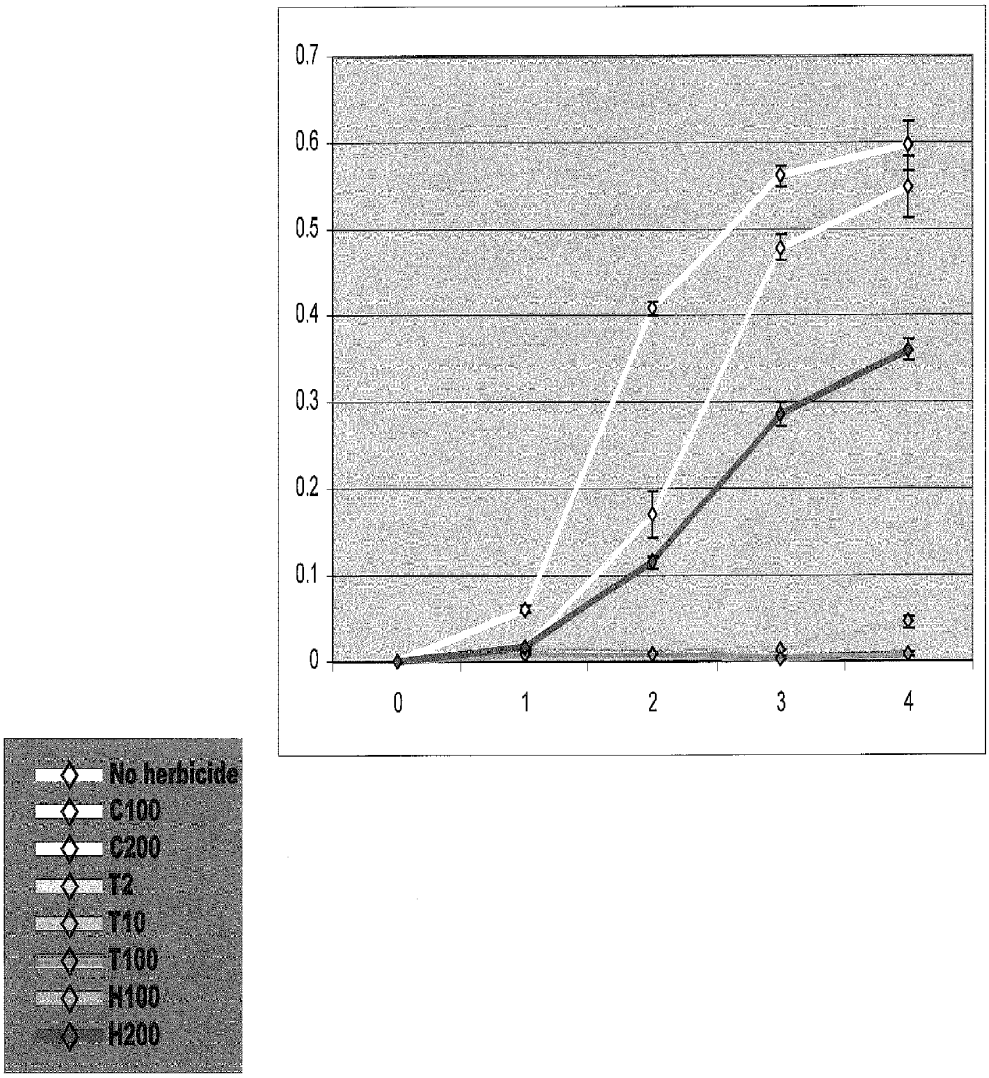


FIGURE 48

I1781L W2027R

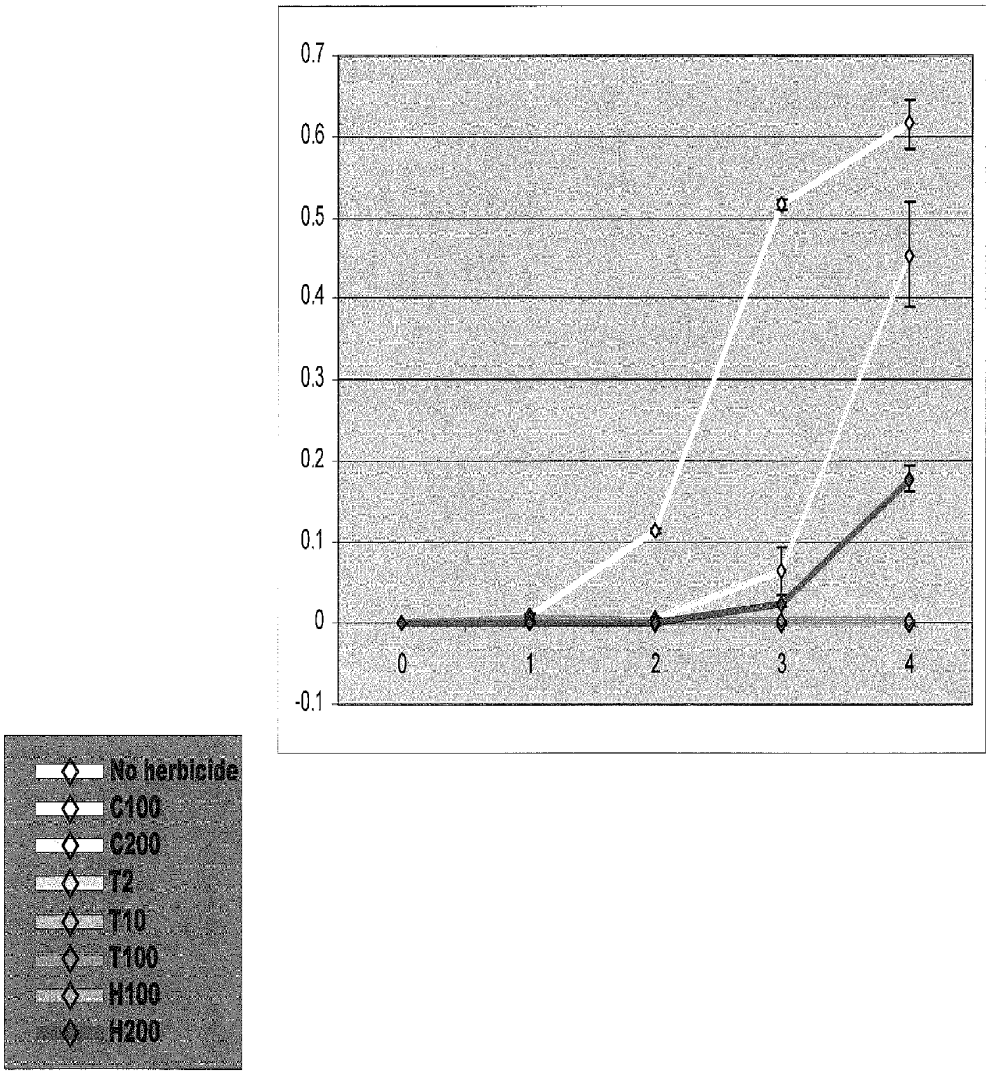


FIGURE 49

D2078G S2079P

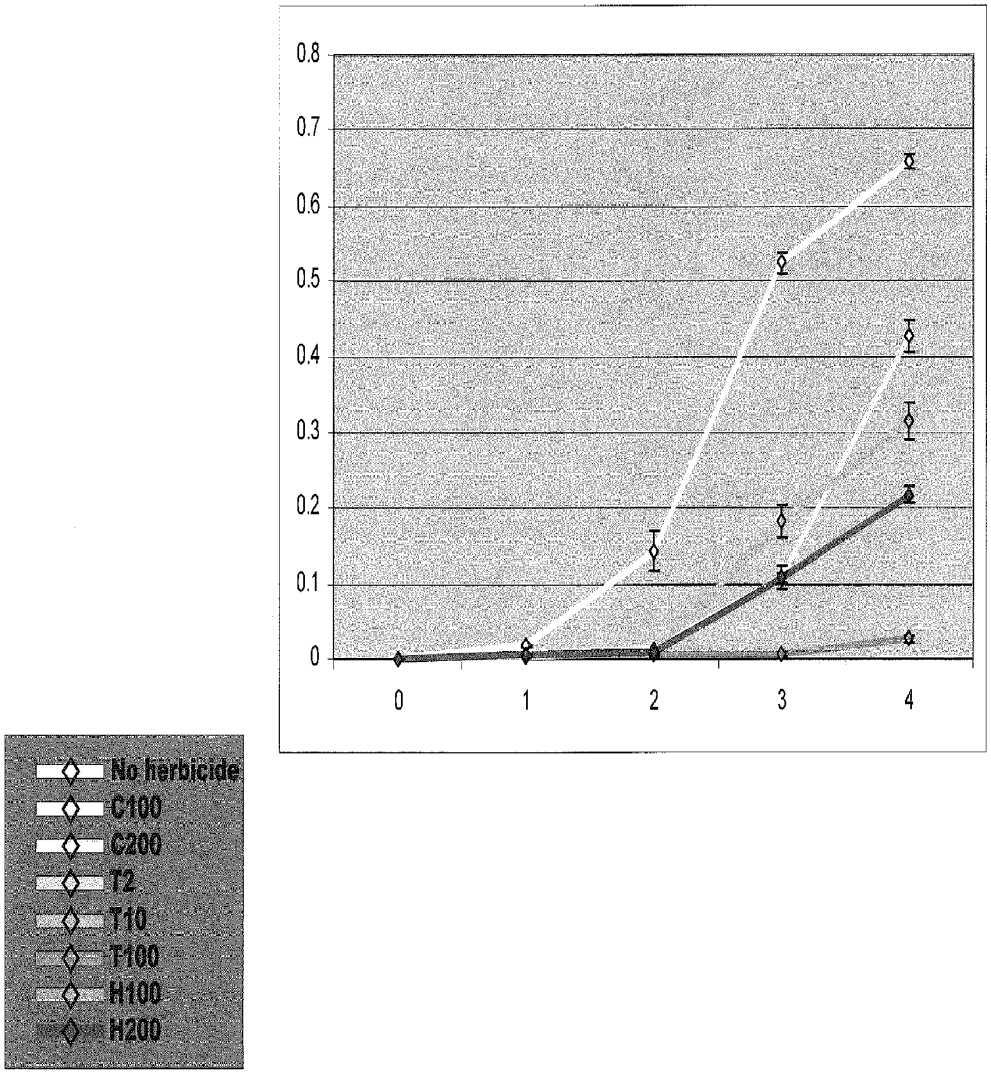


FIGURE 50

D2078G S2079F

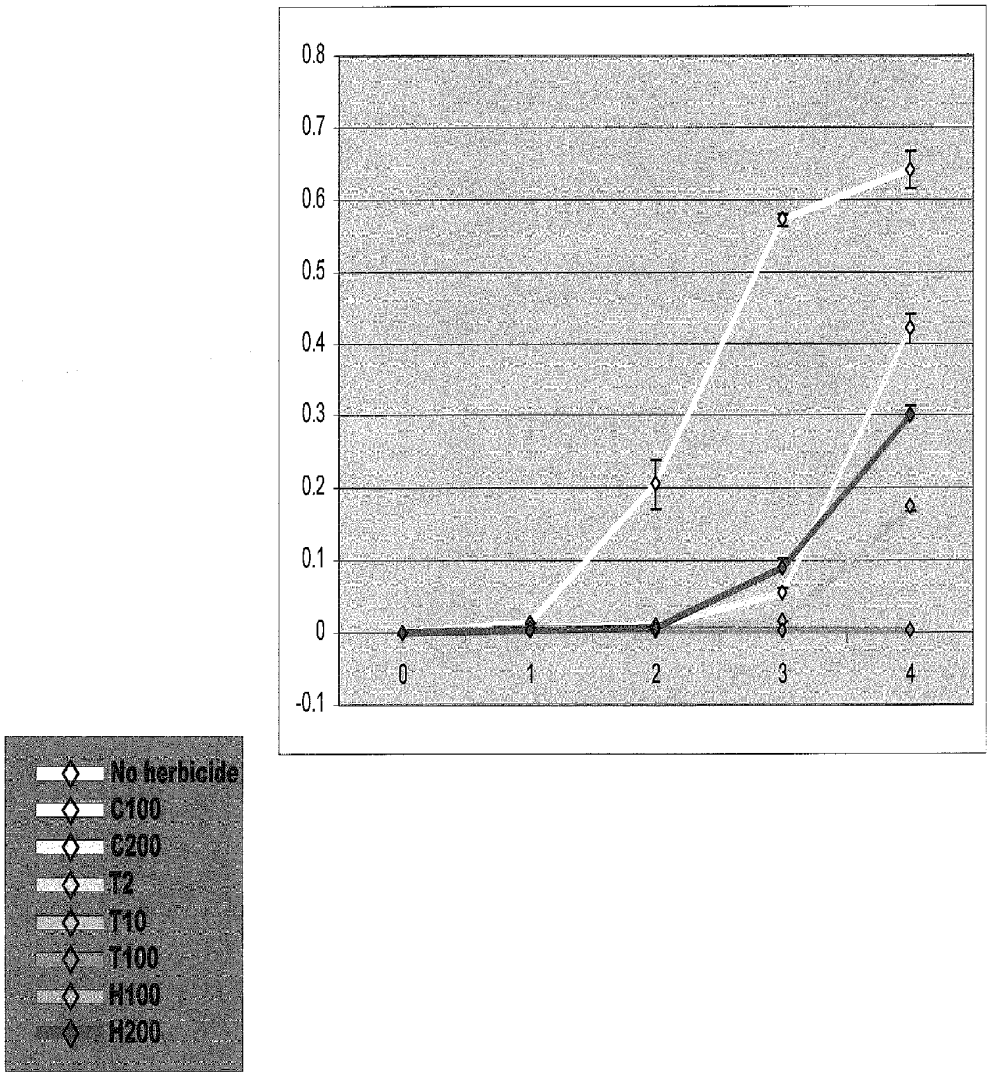
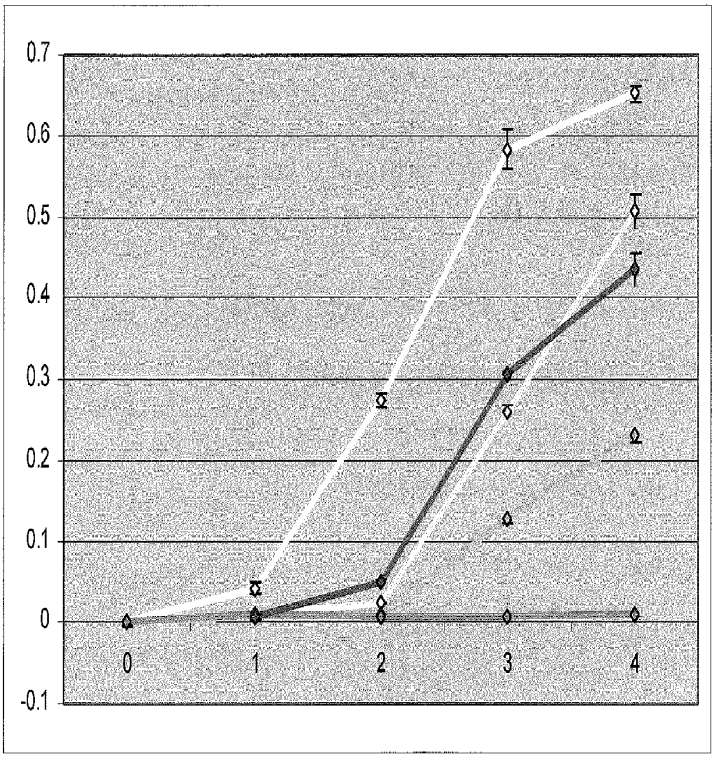


FIGURE 51

E2039G D2078G



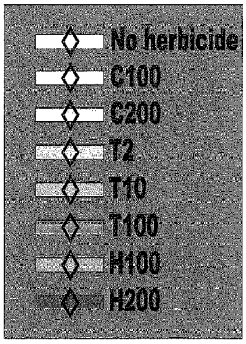


FIGURE 52

C2088K V2098A

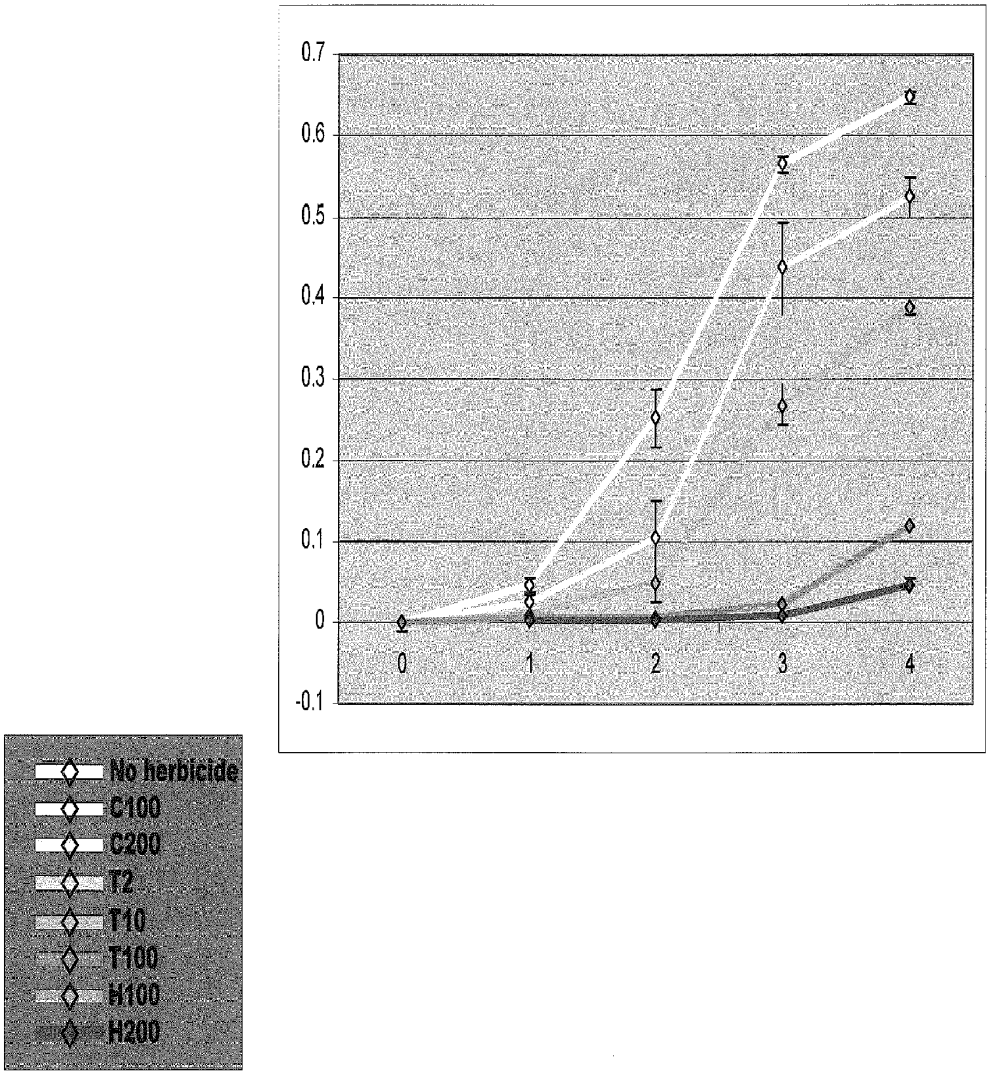


FIGURE 53

C2088L V2098G

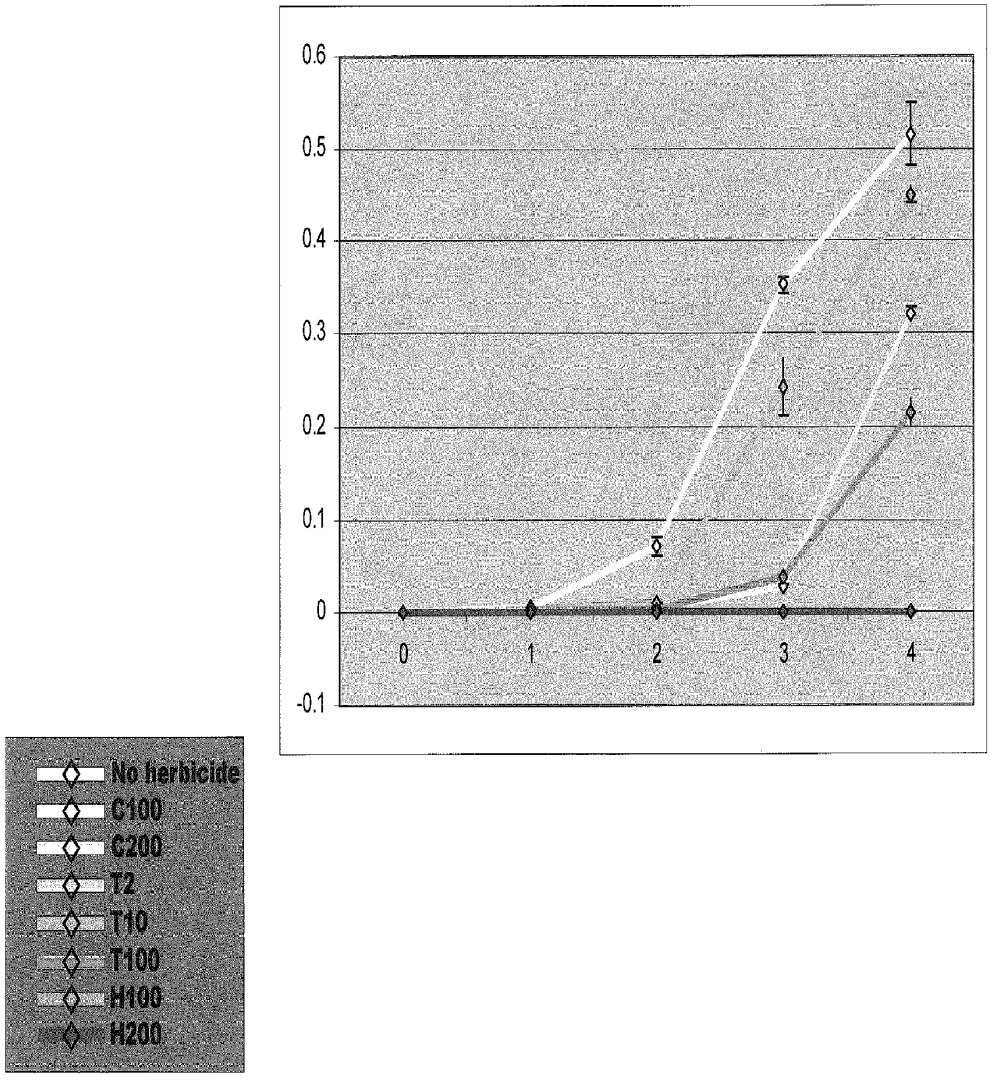
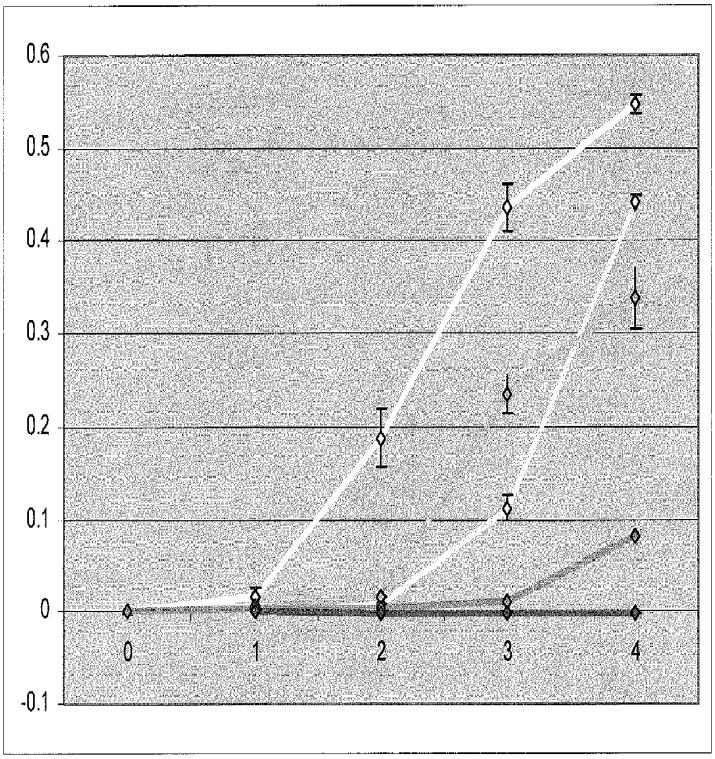


FIGURE 54

C2088S V2098G



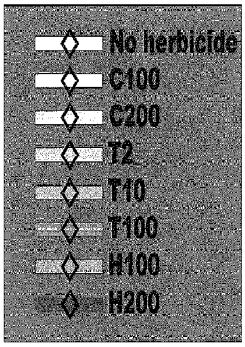
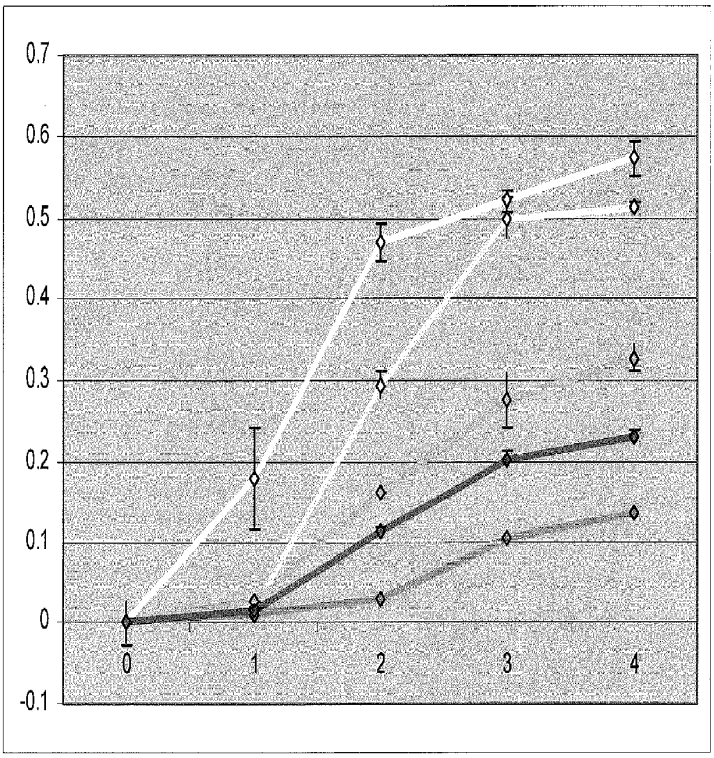


FIGURE 55

C2088L V2098A



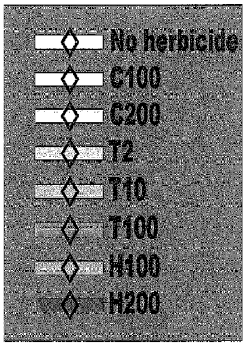


FIGURE 56

C2088V V2098G

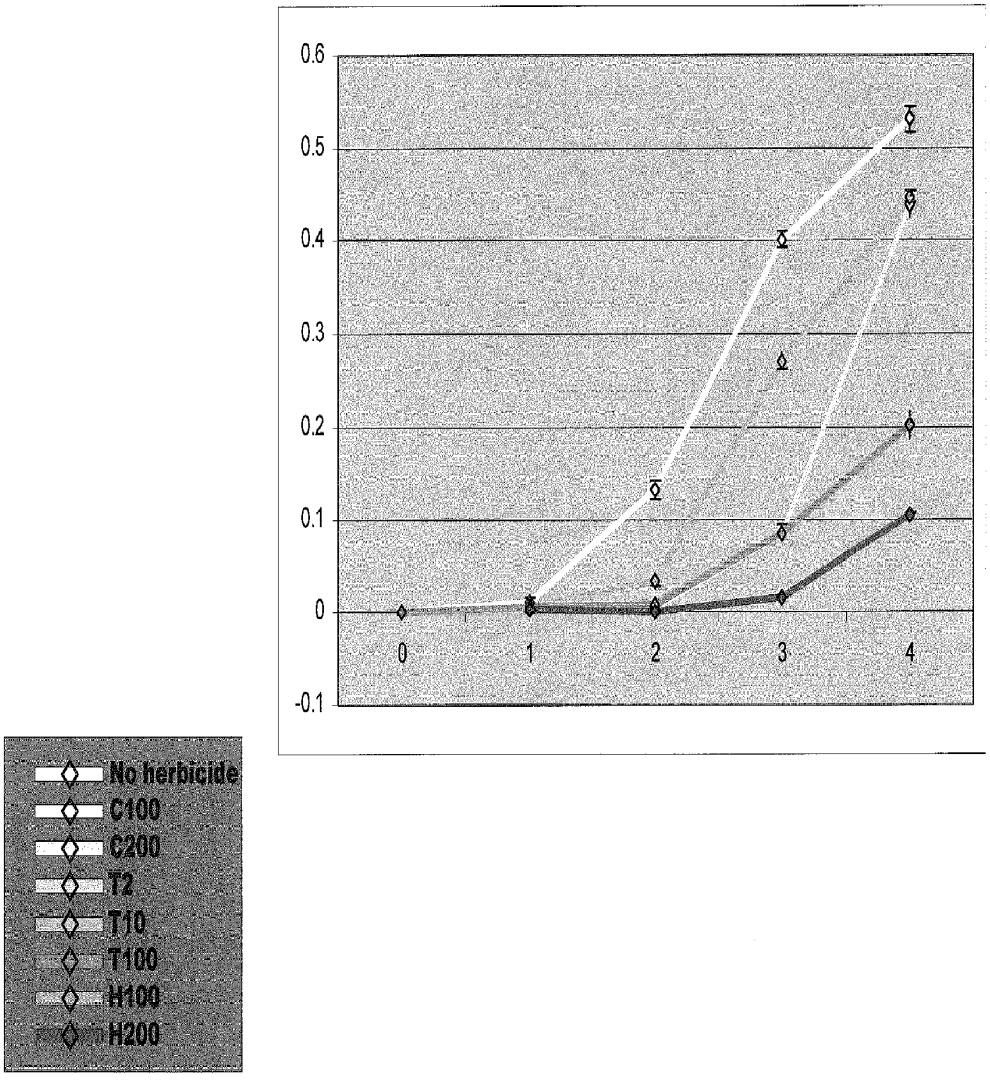


FIGURE 57

C2088H V2098A

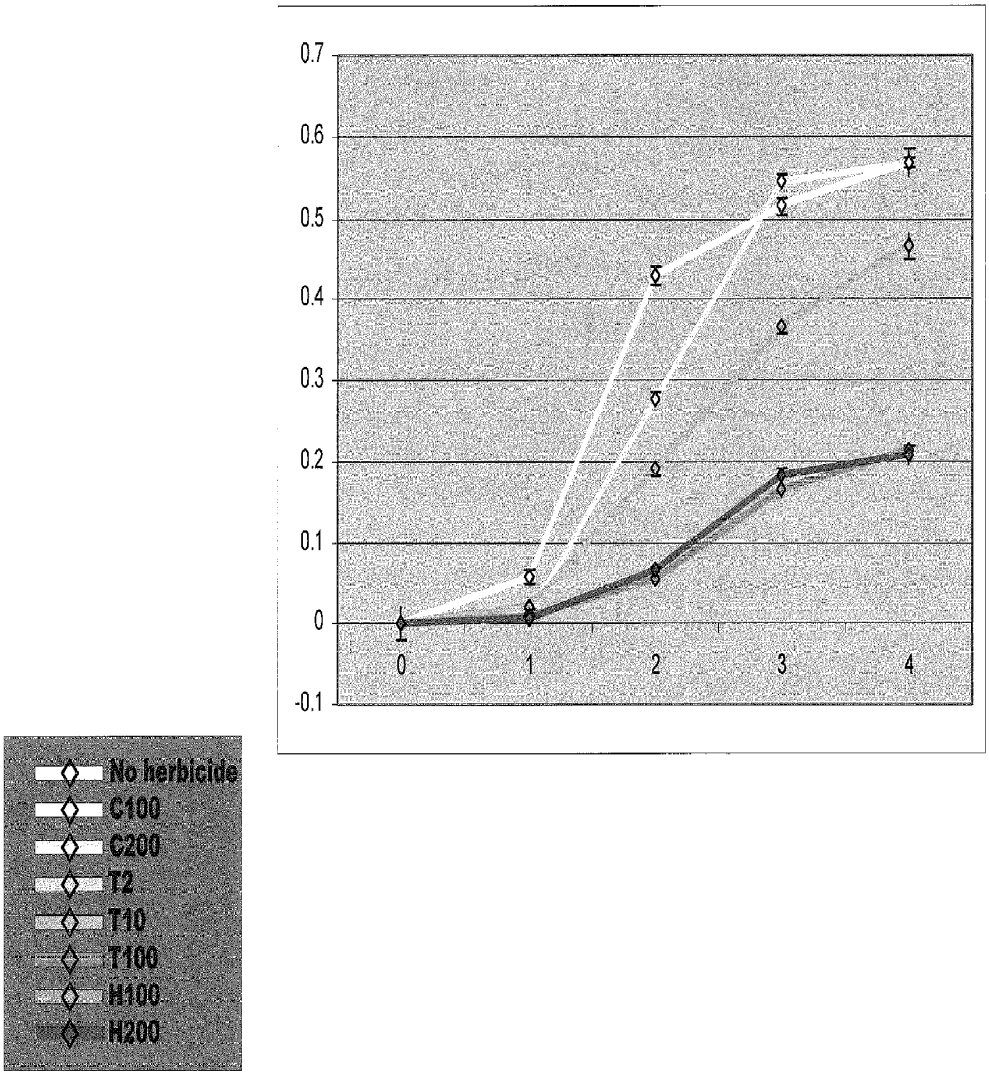


FIGURE 58

C2088G V2098G

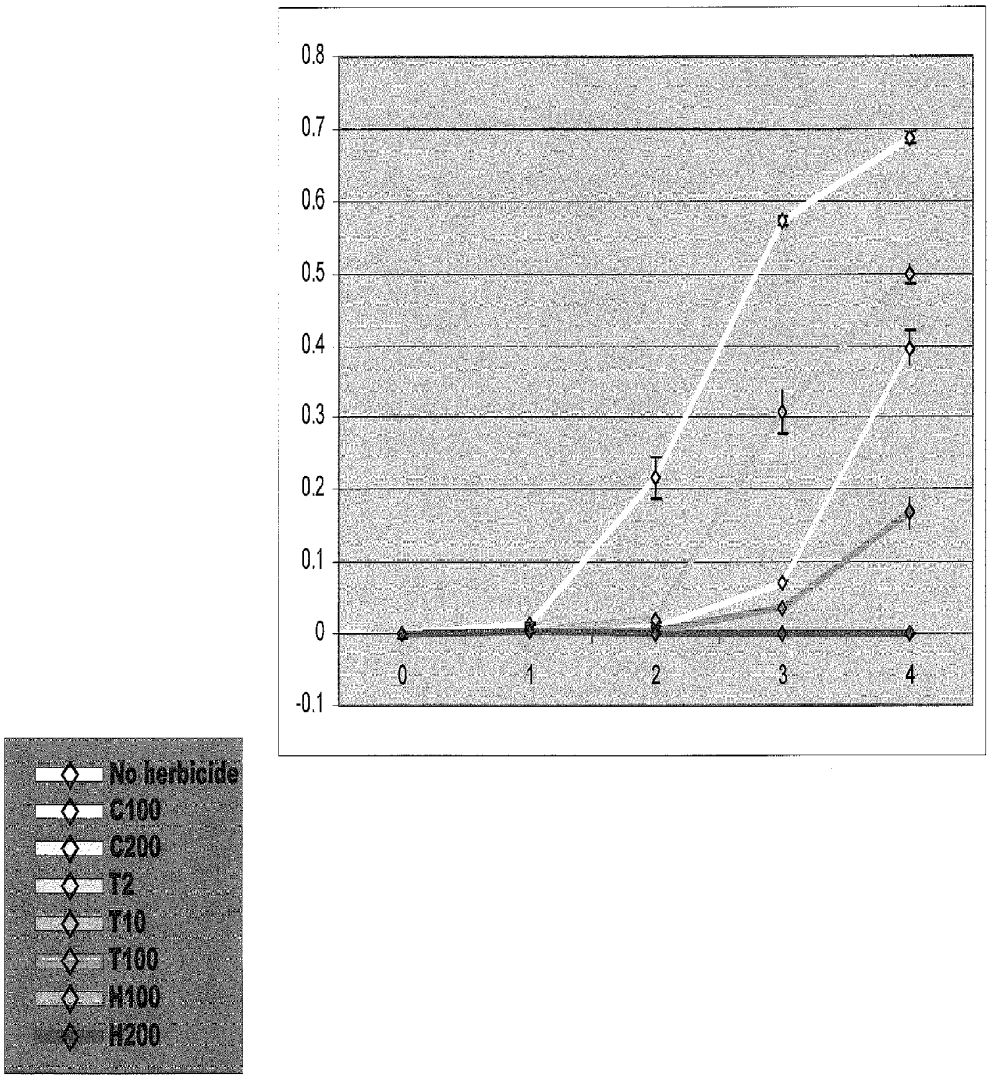


FIGURE 59

C2088T V2098G

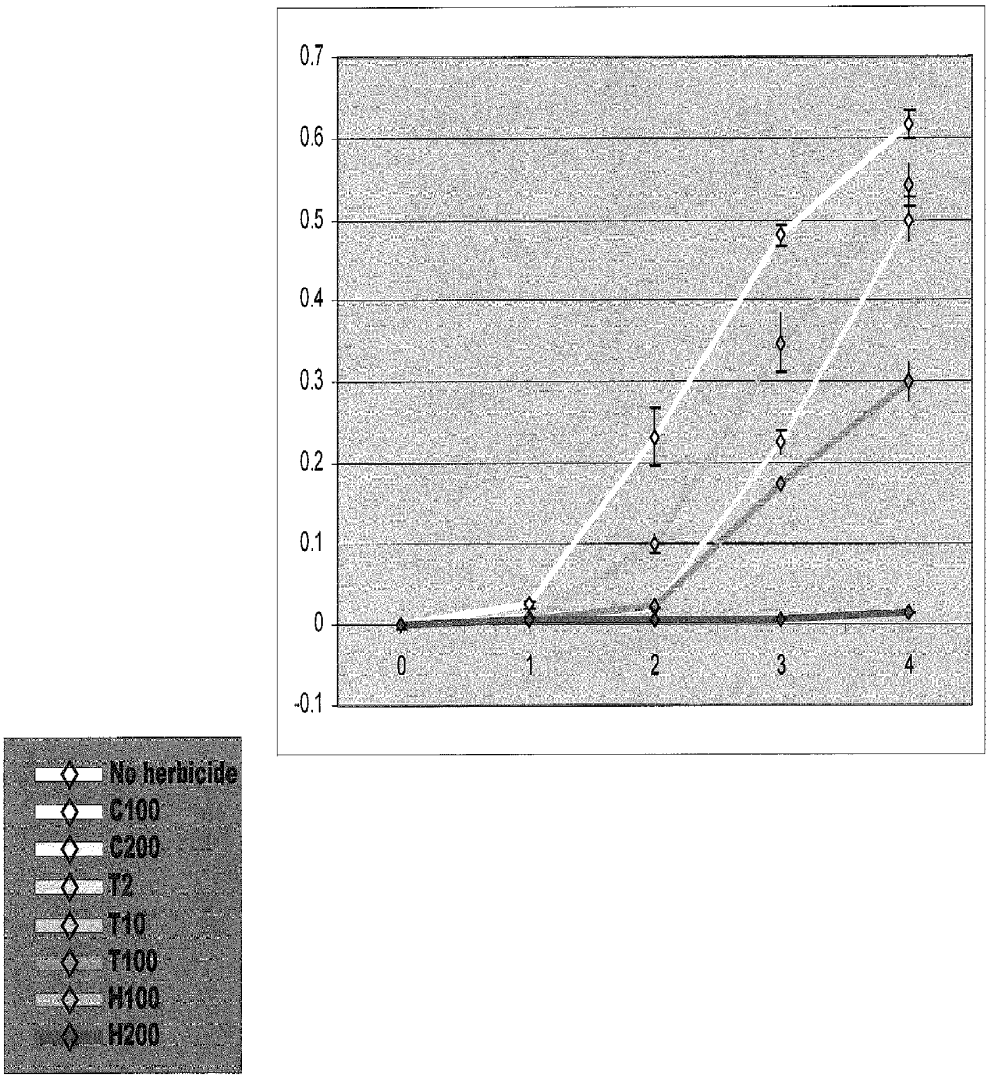
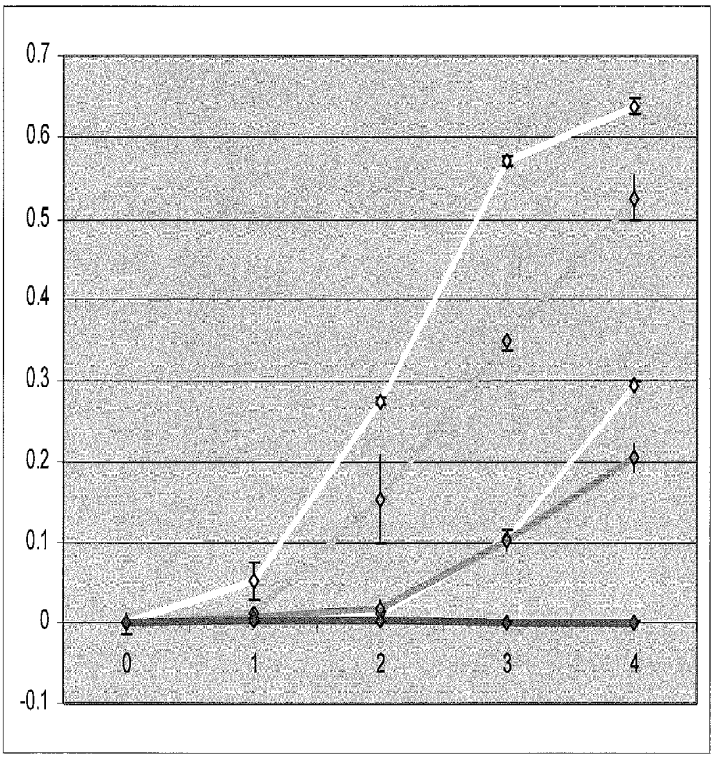


FIGURE 60

V2049T D2078G



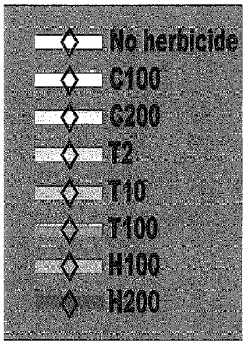


FIGURE 61

V2049L V2098A

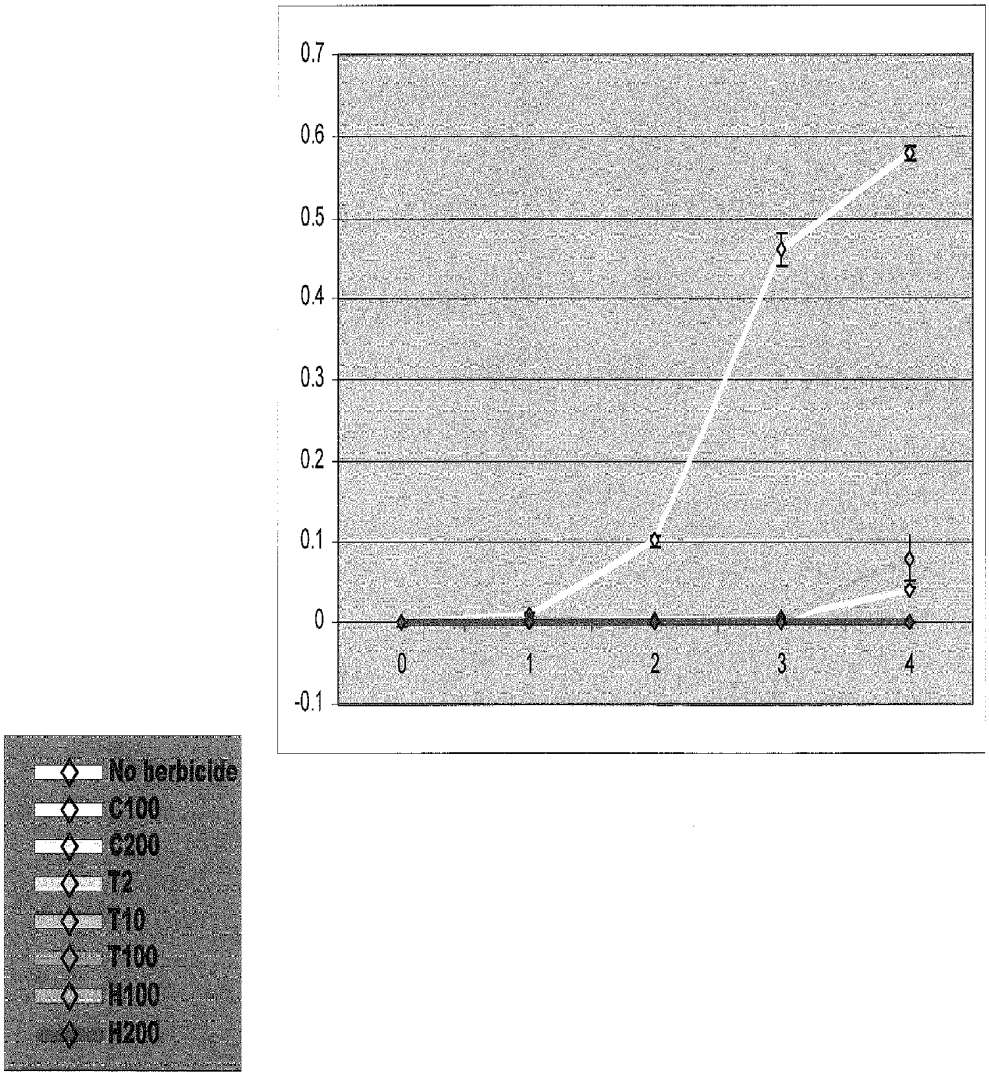
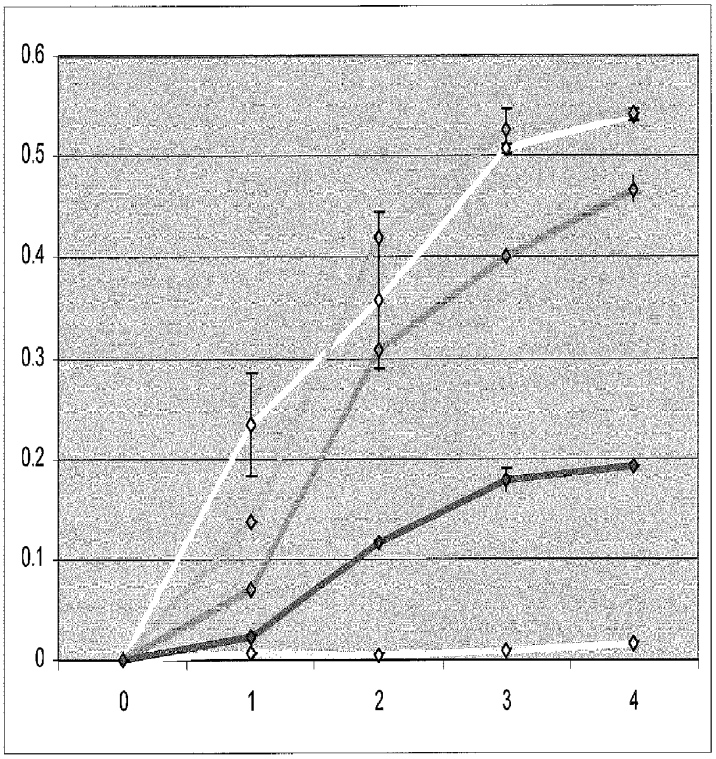


FIGURE 62

V2075M K2080E



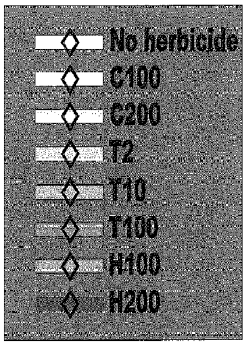


FIGURE 63

V2075M K2095E

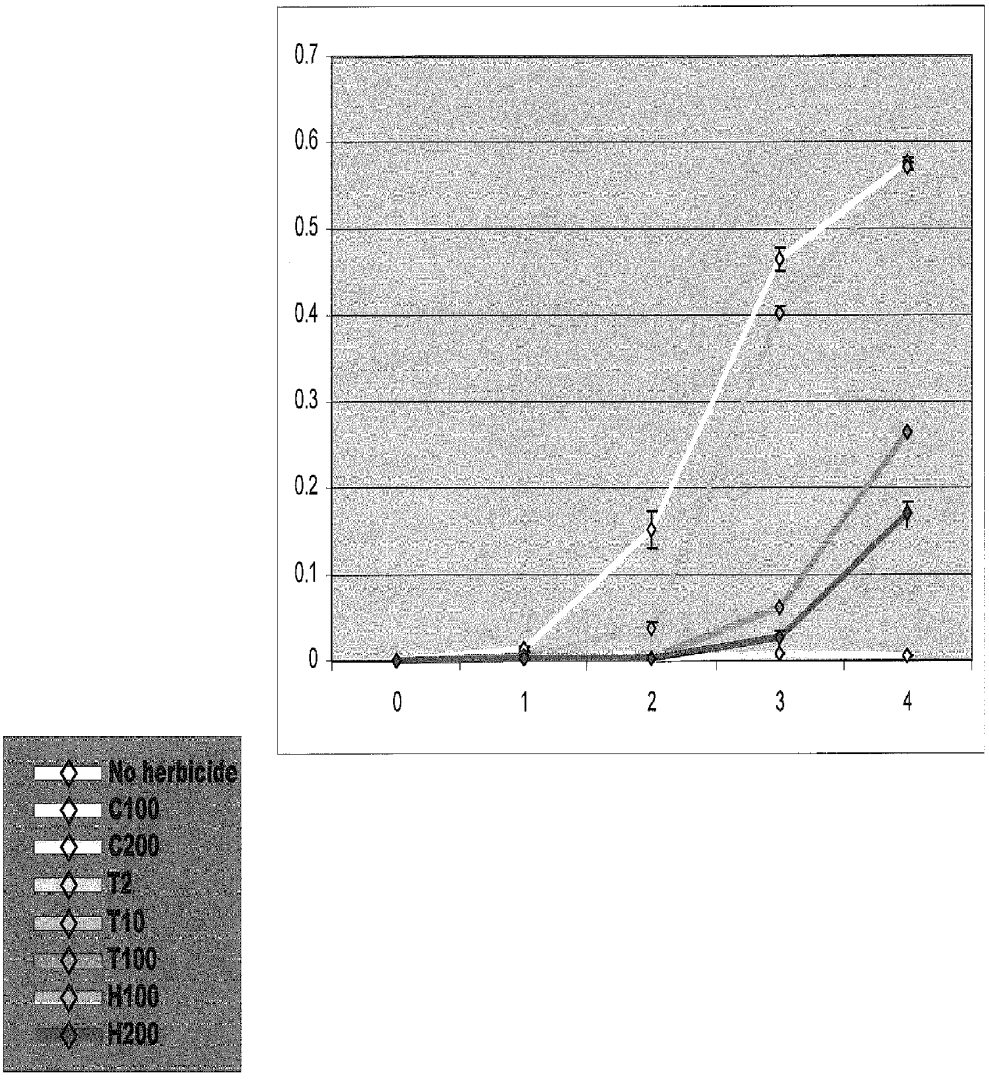


FIGURE 64

D2078G C2088G

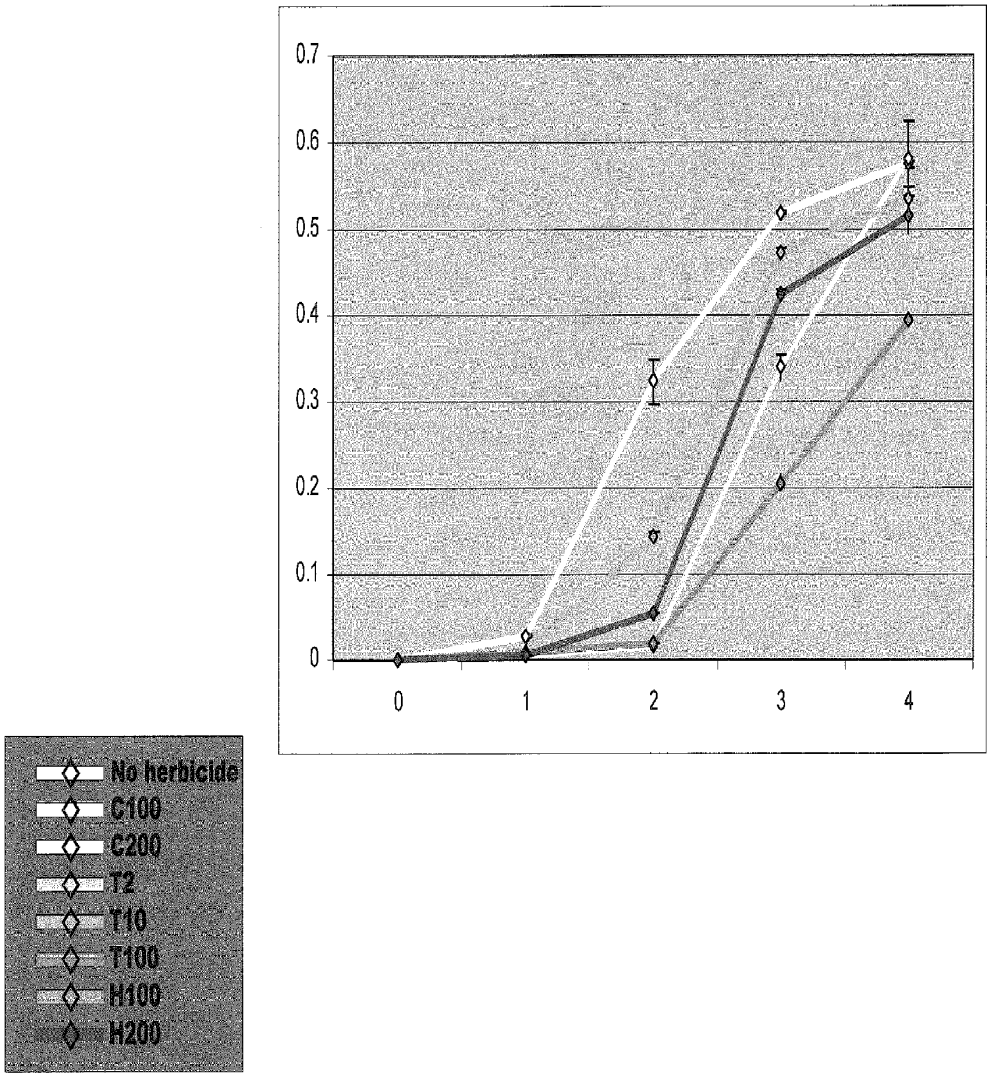


FIGURE 65

I2041V D2078G

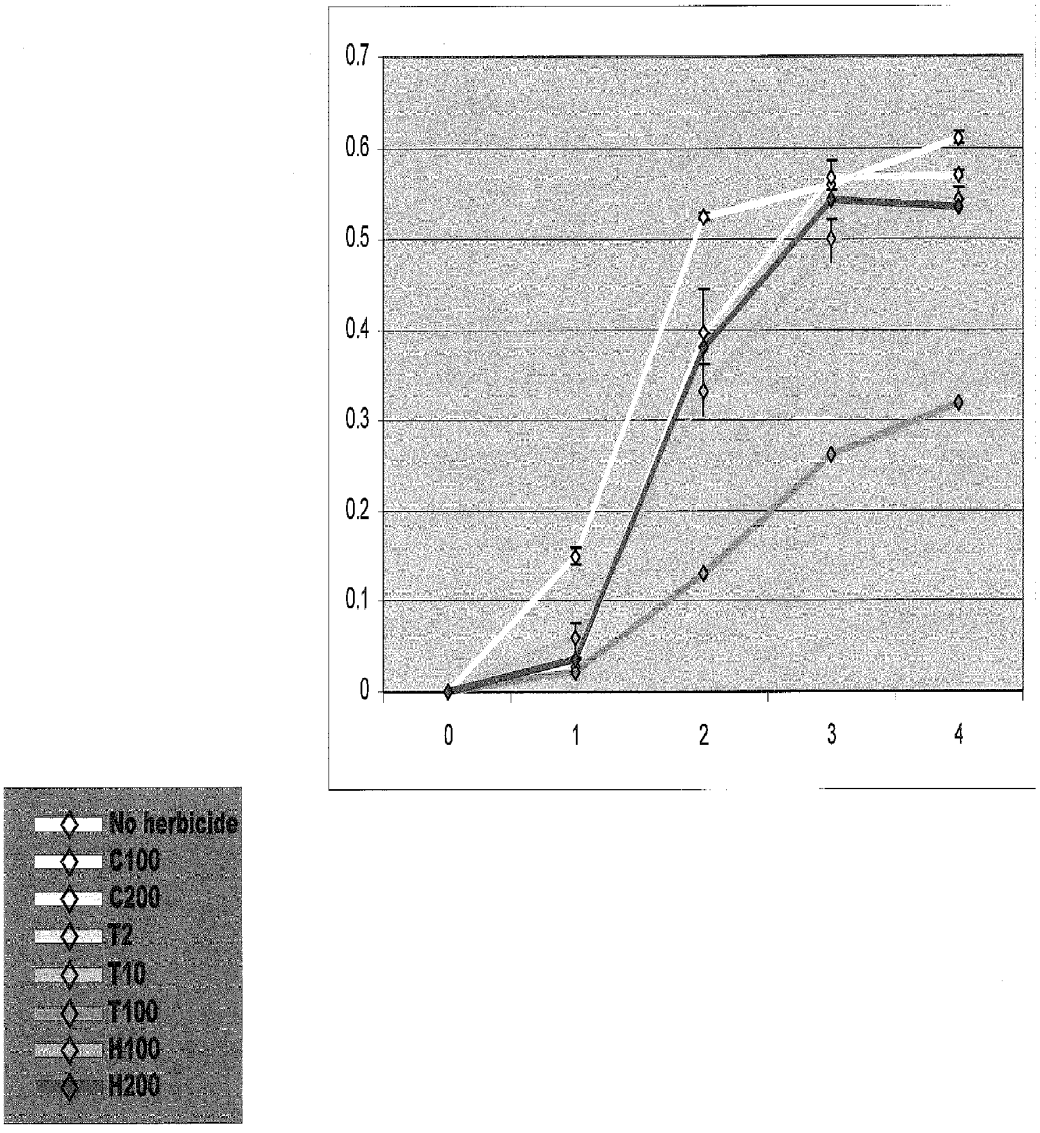


FIGURE 66

C2088W V2098C

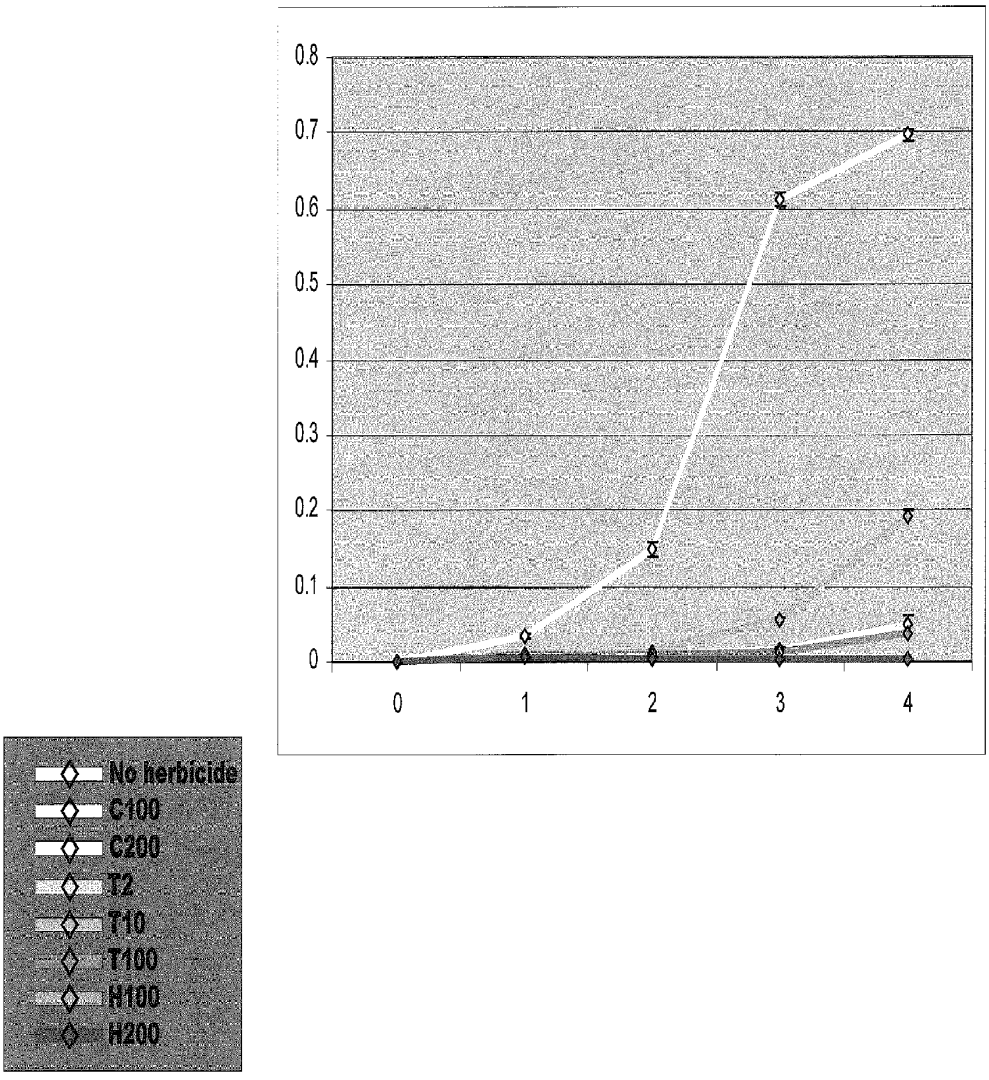


FIGURE 67

C2088H V2098G

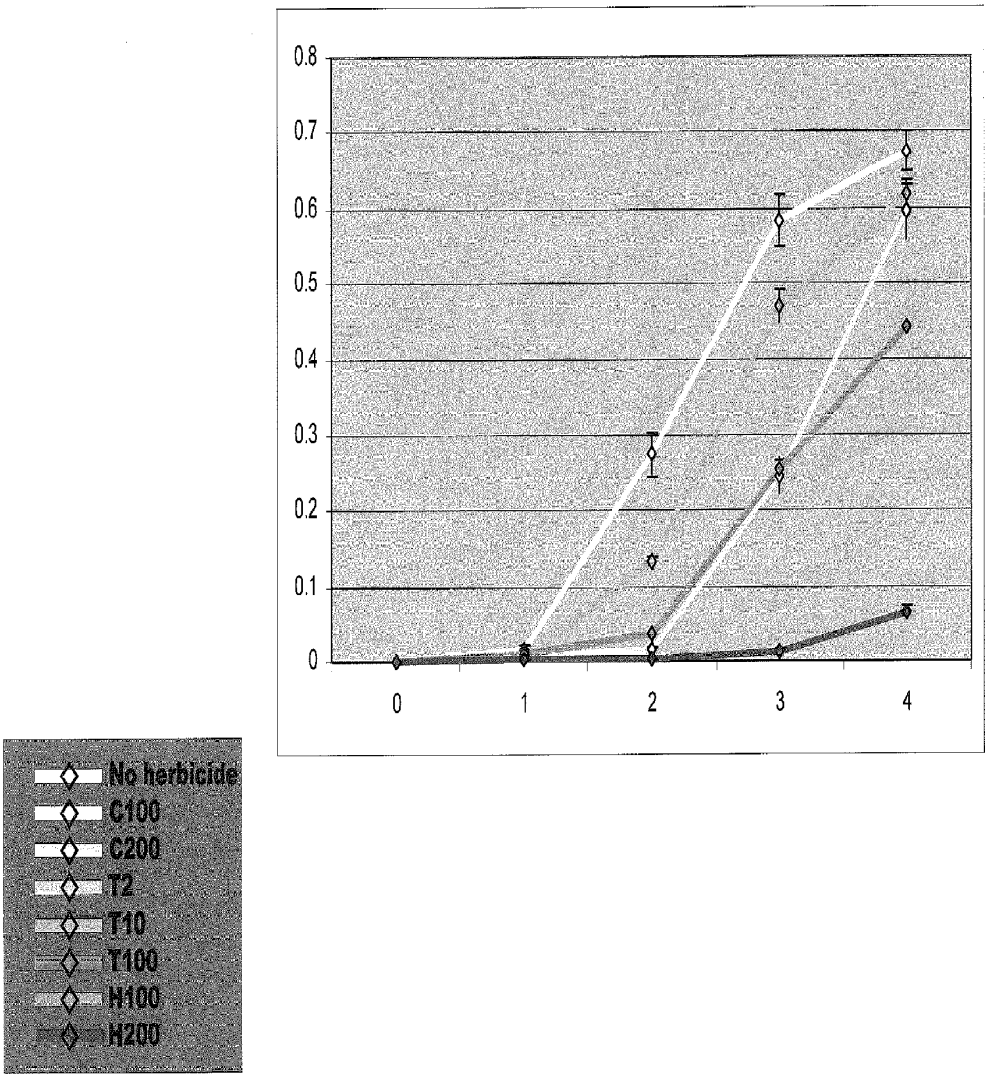


FIGURE 68

C2088F V2098A

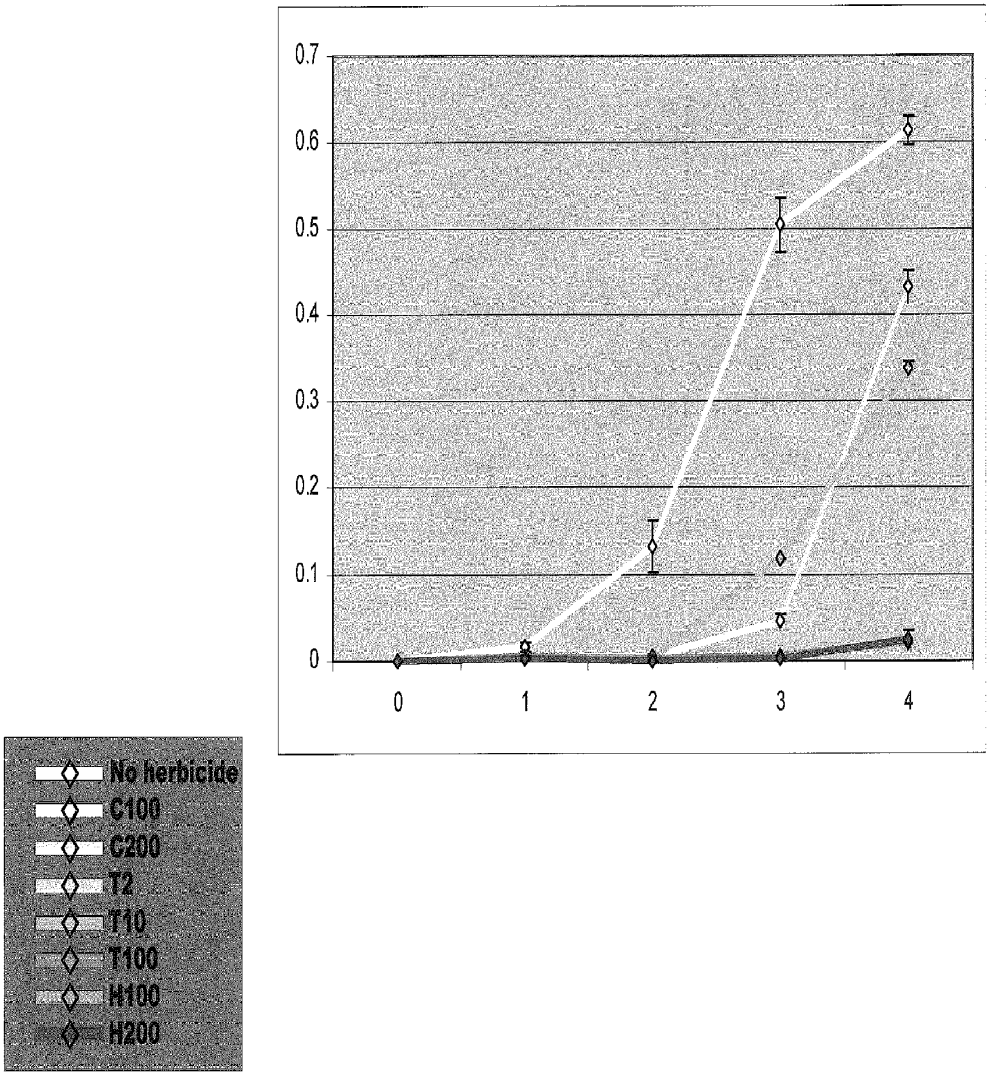


FIGURE 69

W1999G V2098A

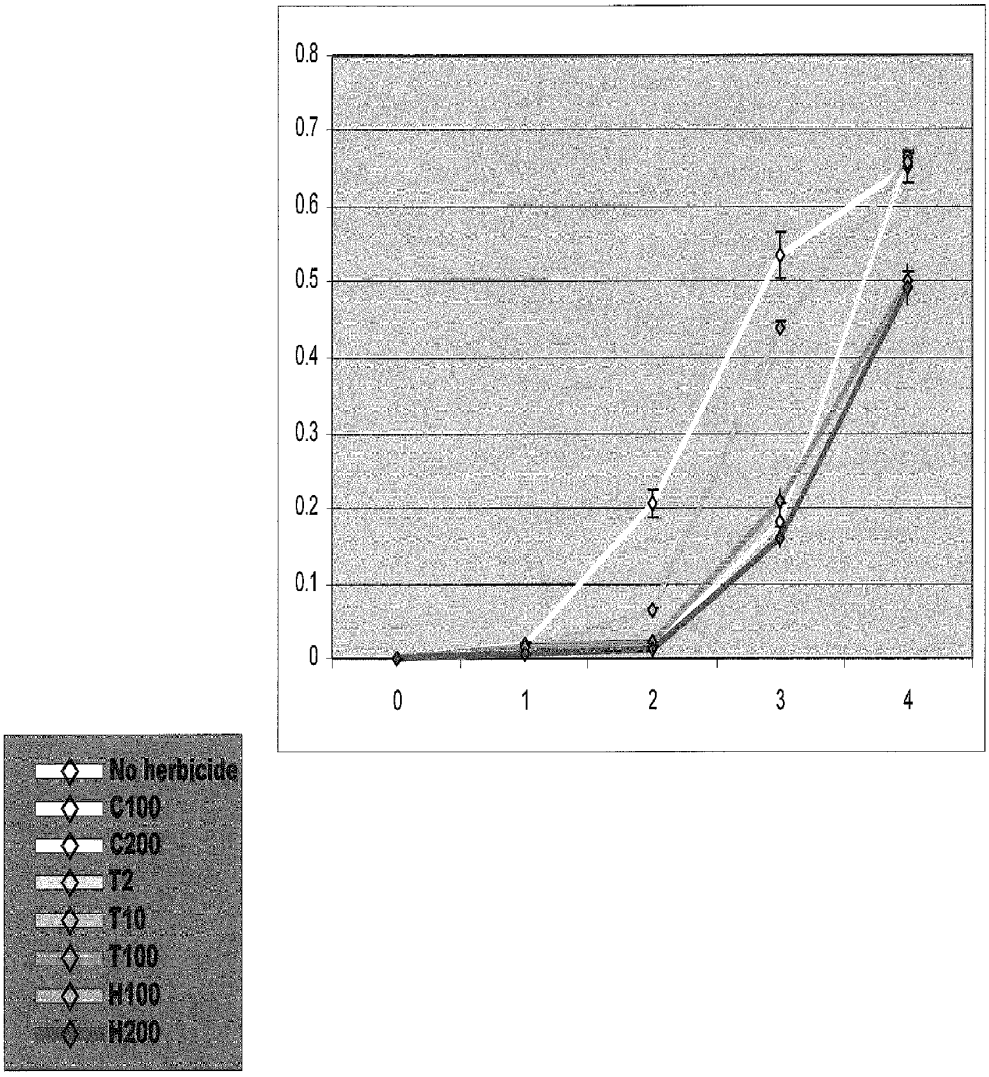


FIGURE 70

W1999G V2075L

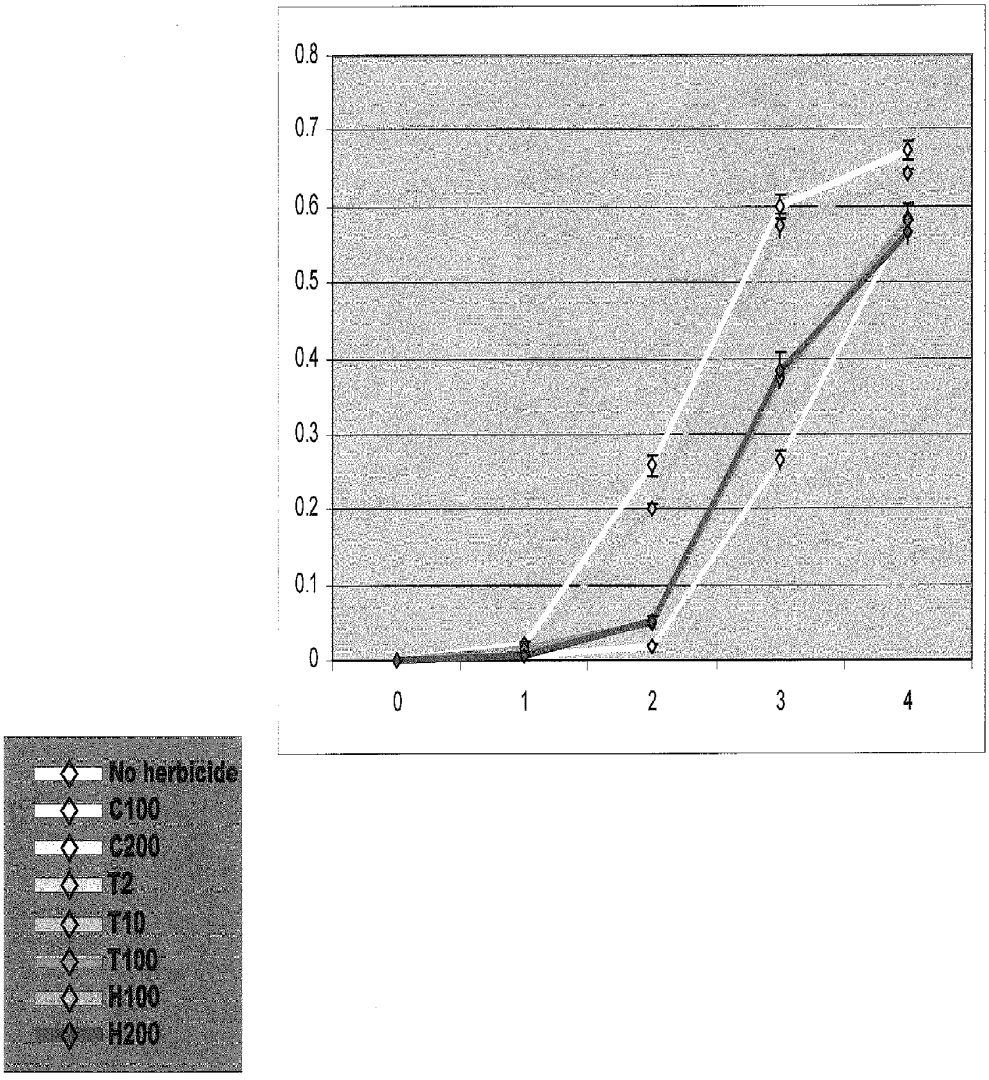


FIGURE 71

I1781L V2075L

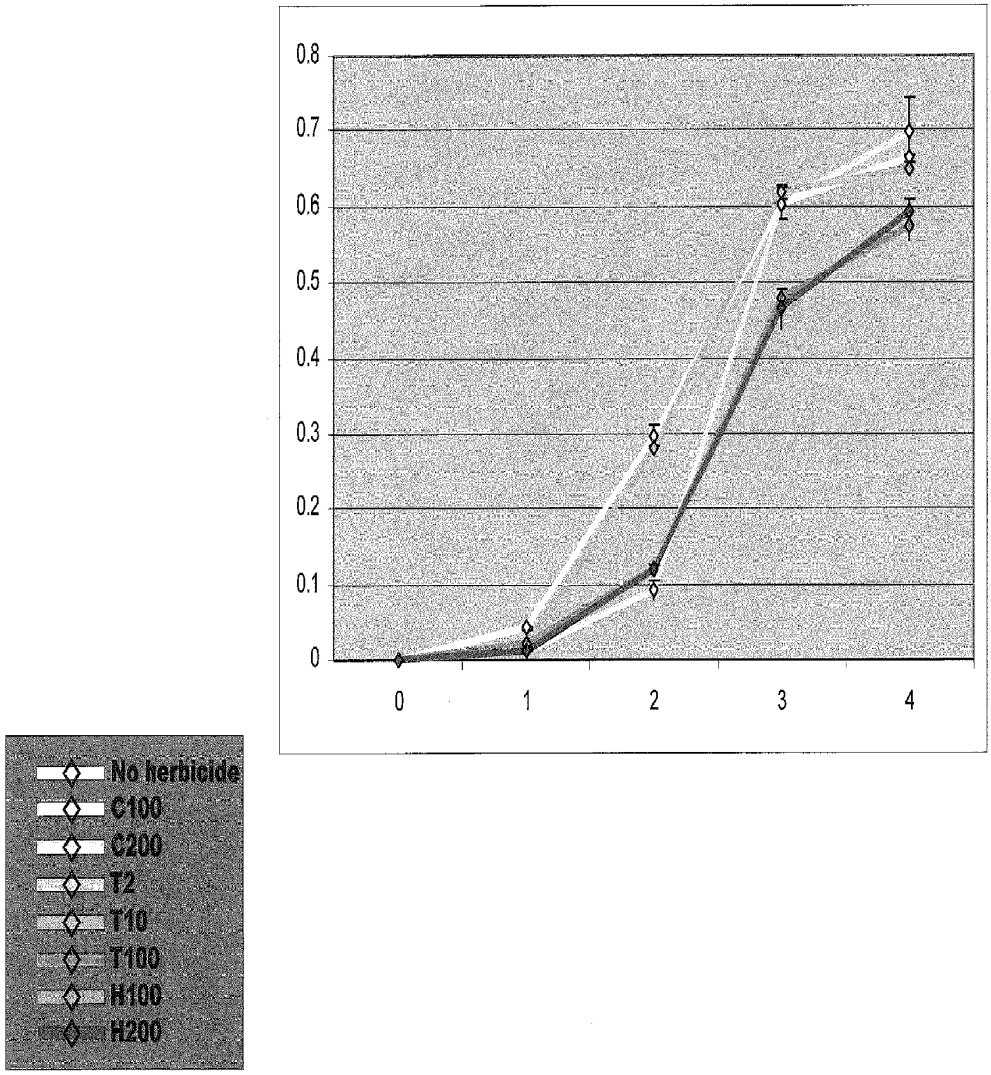


FIGURE 72

I1781L V2075L

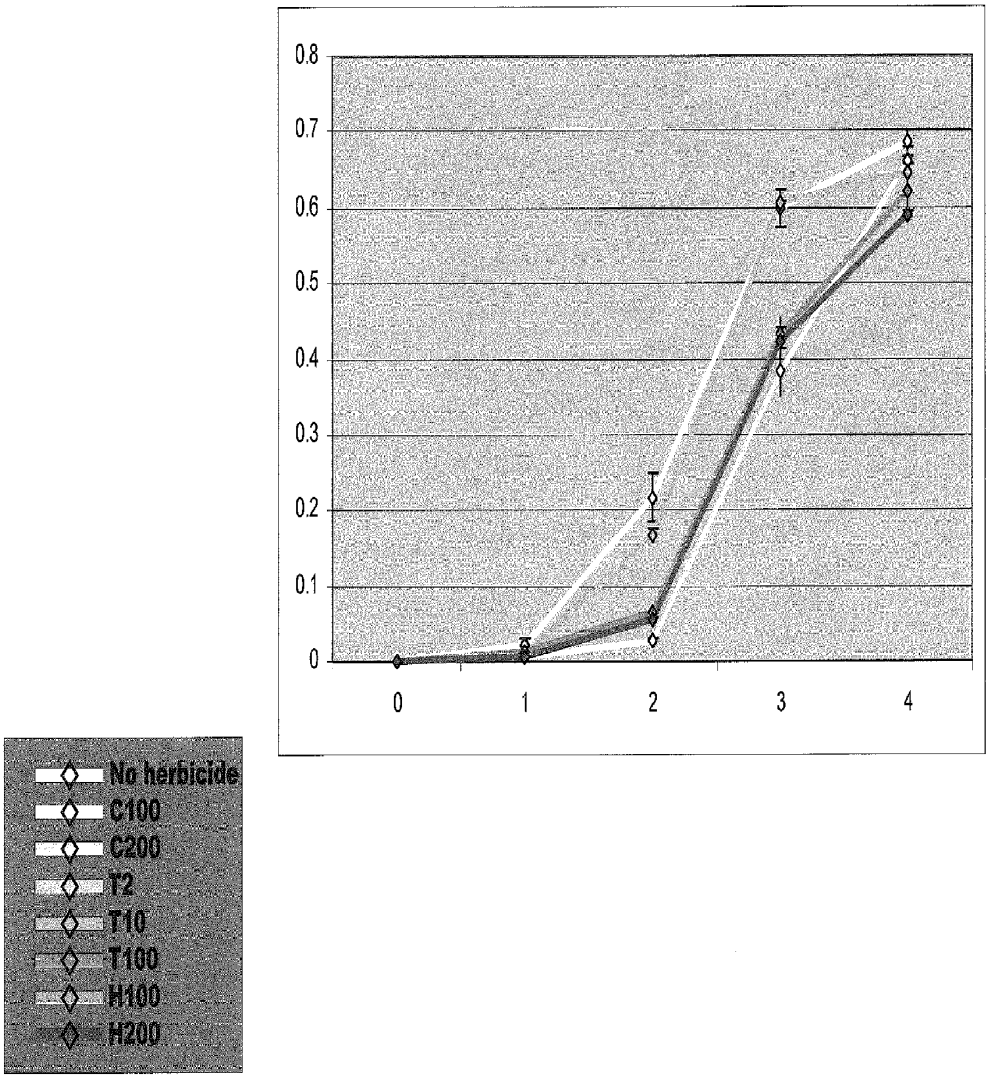


FIGURE 73

I1781L V2075L

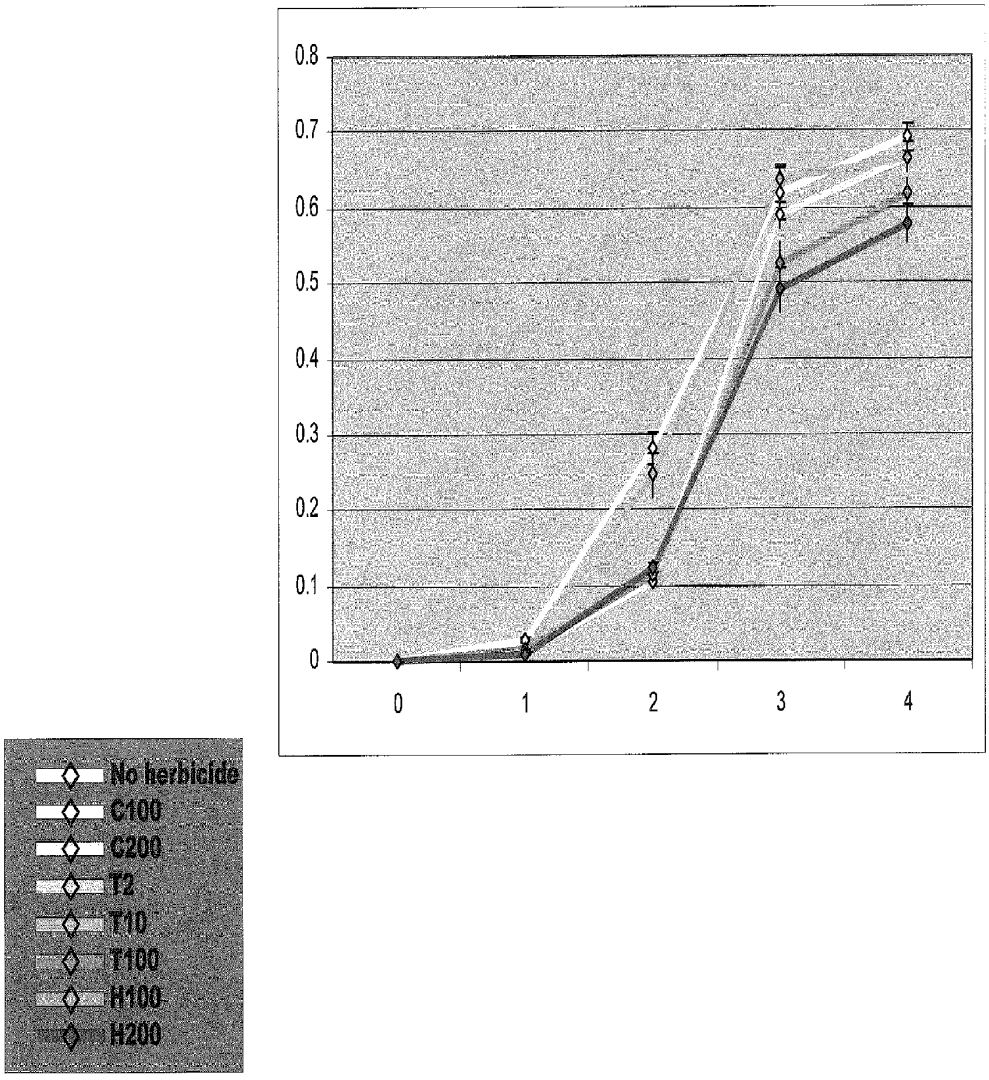


FIGURE 74

I1781L V2049F

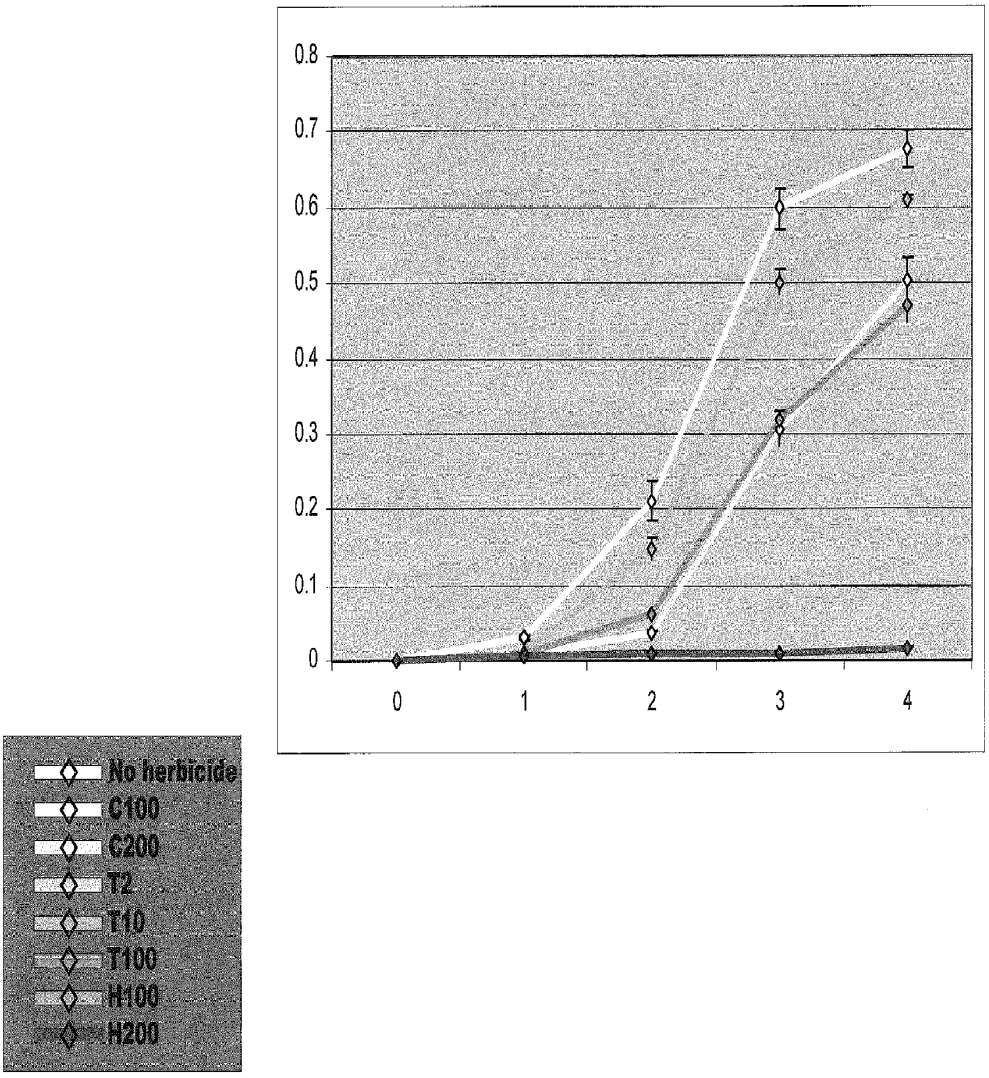


FIGURE 75

W1999G V2075I

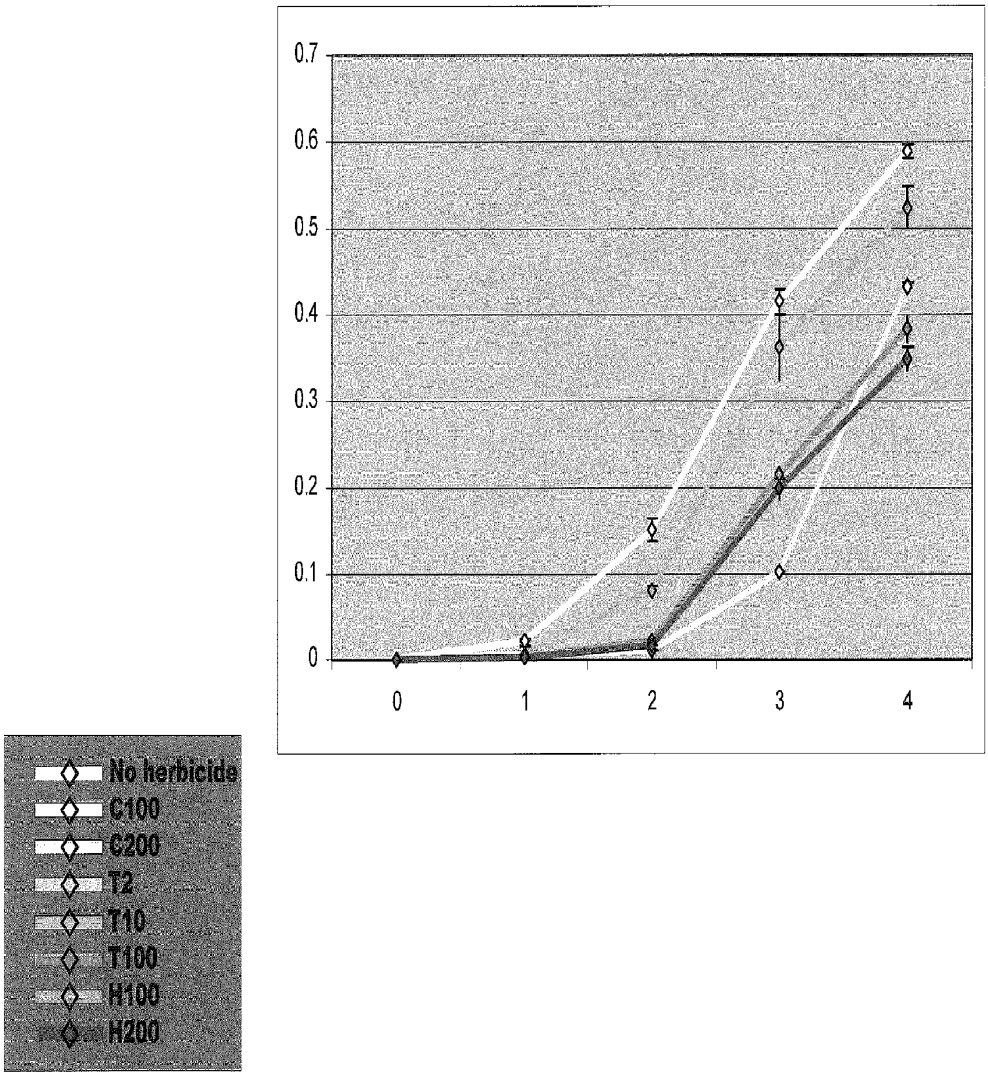


FIGURE 76

W1999G V2075L

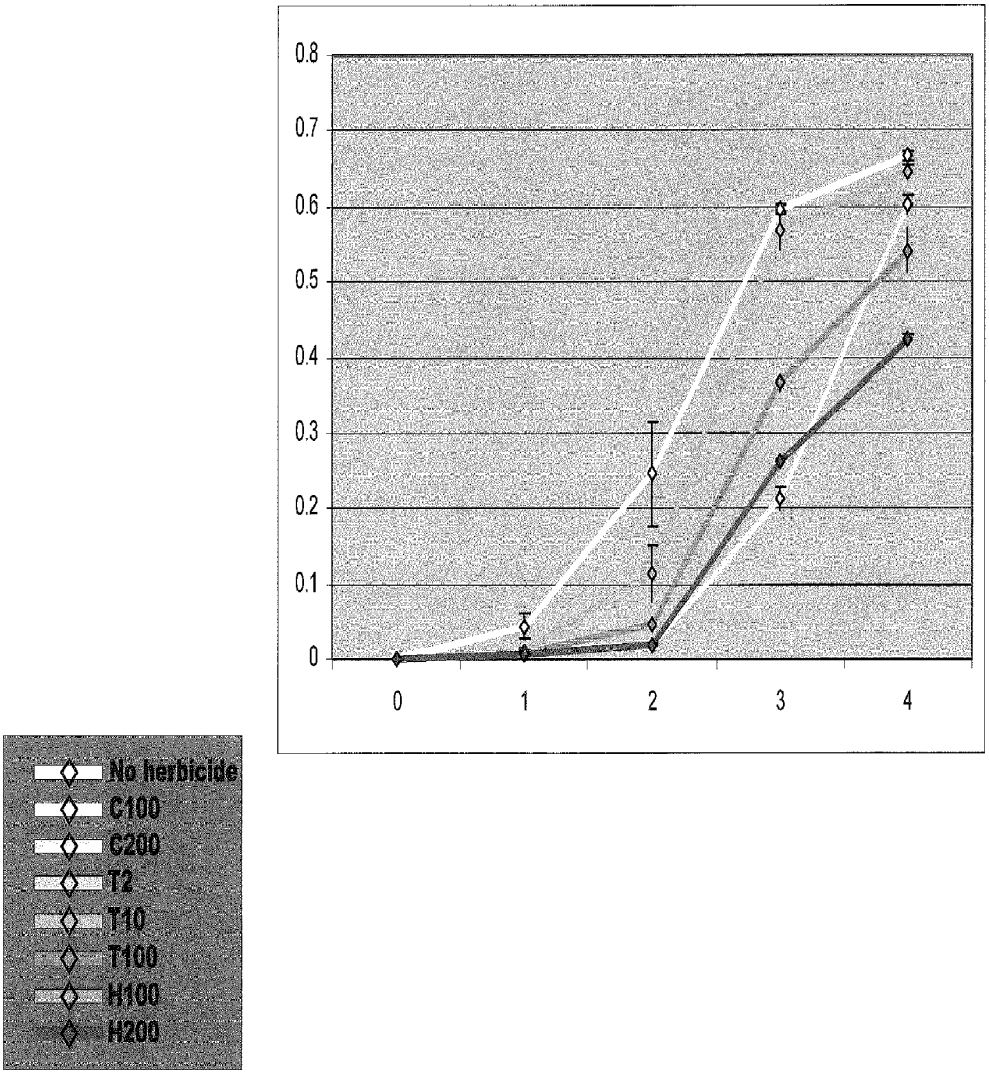


FIGURE 77

W1999G V2075L

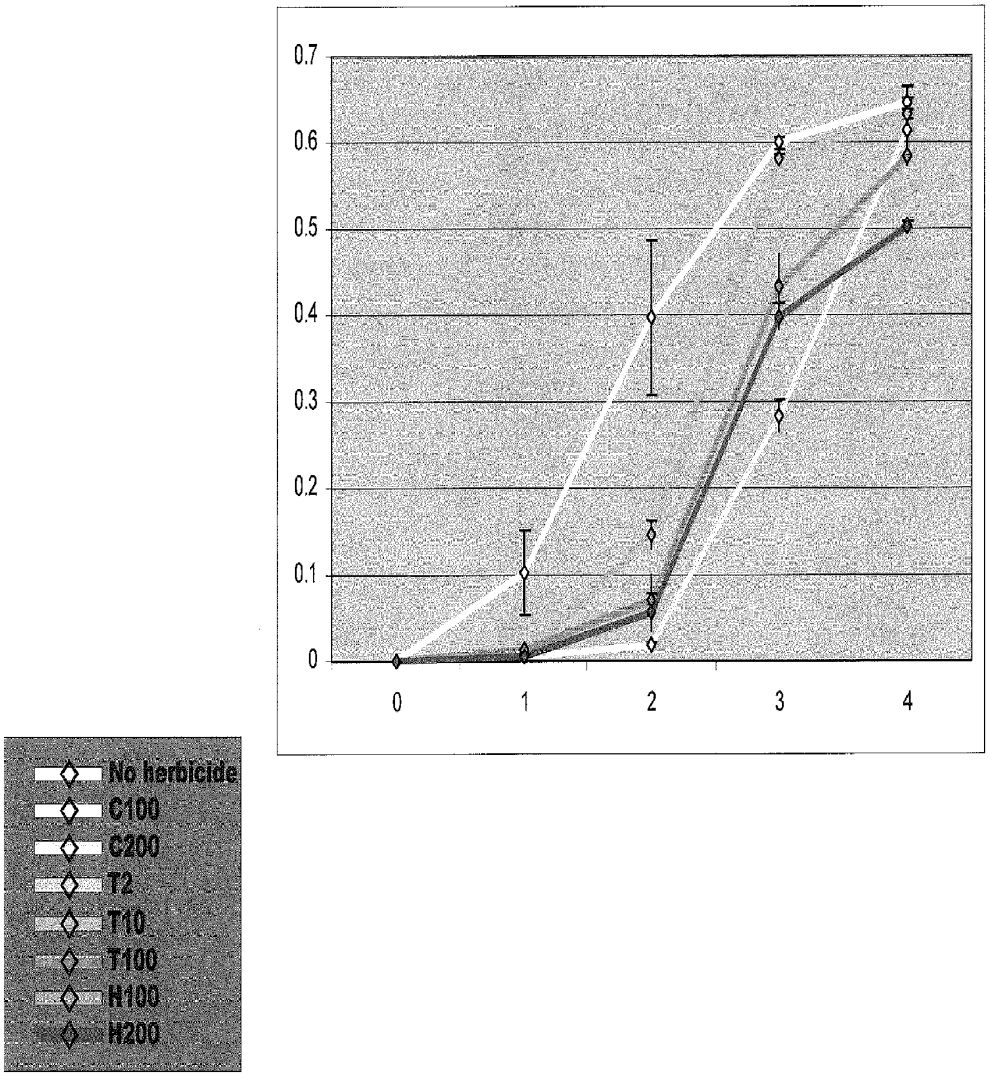


FIGURE 78

V1864F W1999G

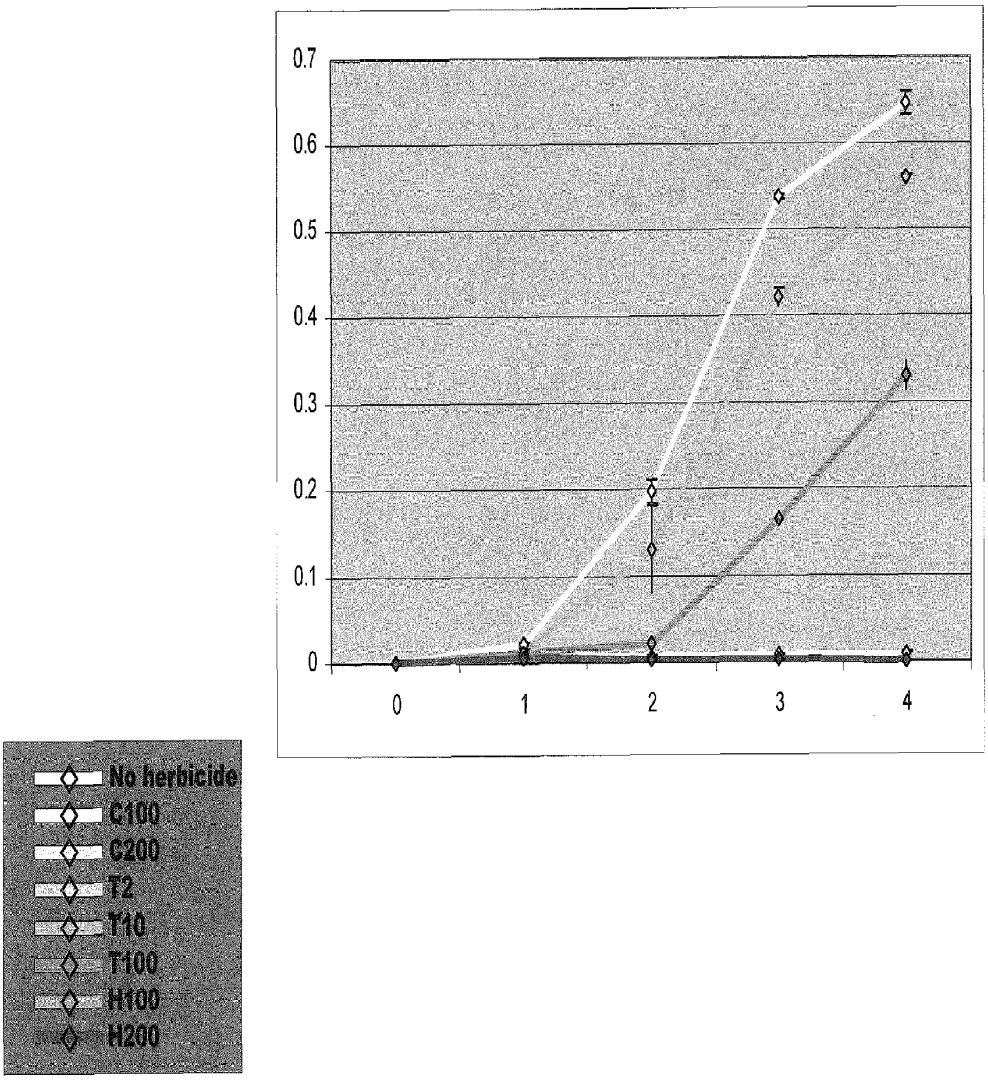


FIGURE 79

W1999G V2098A

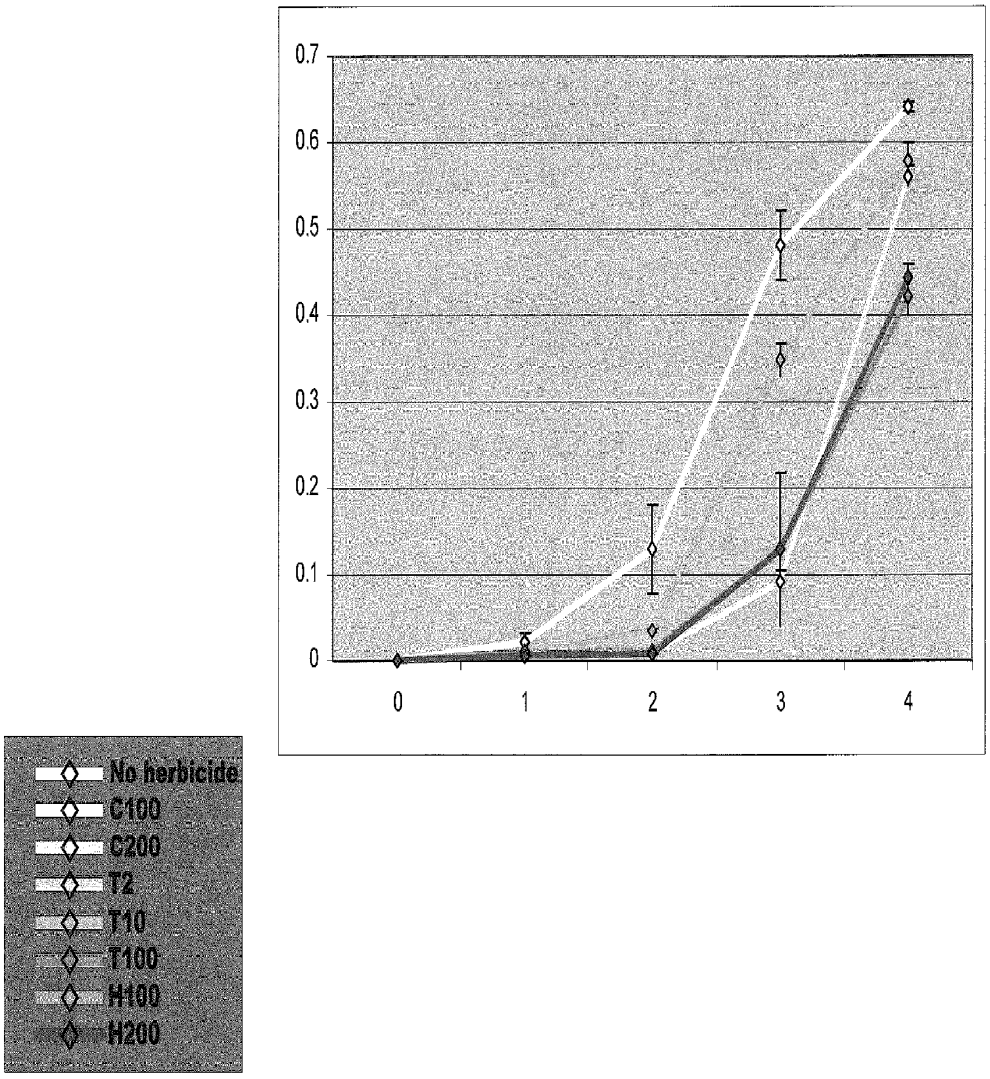
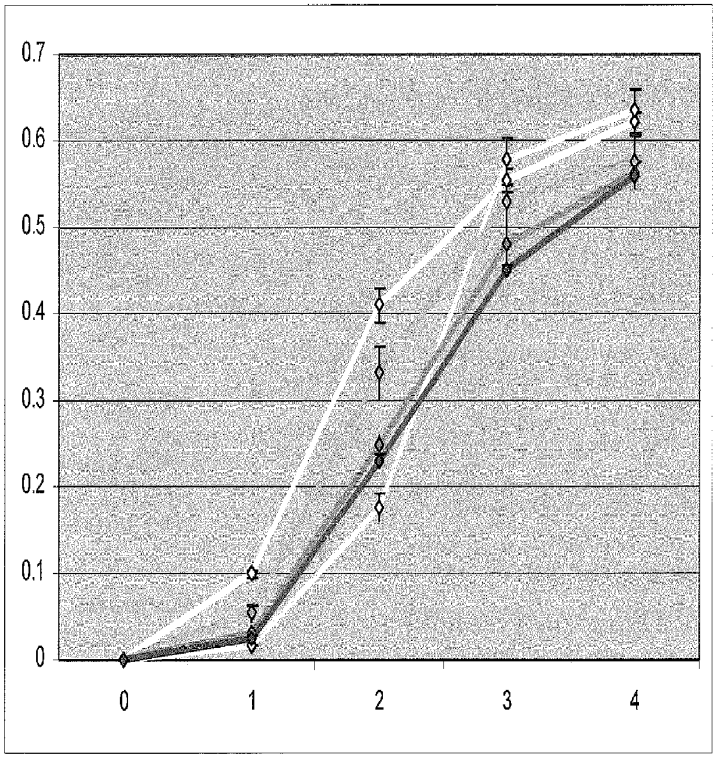


FIGURE 80

W1999G V2098A



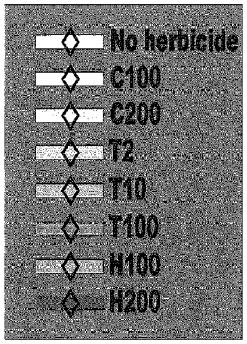


FIGURE 81

I1781L V2098A

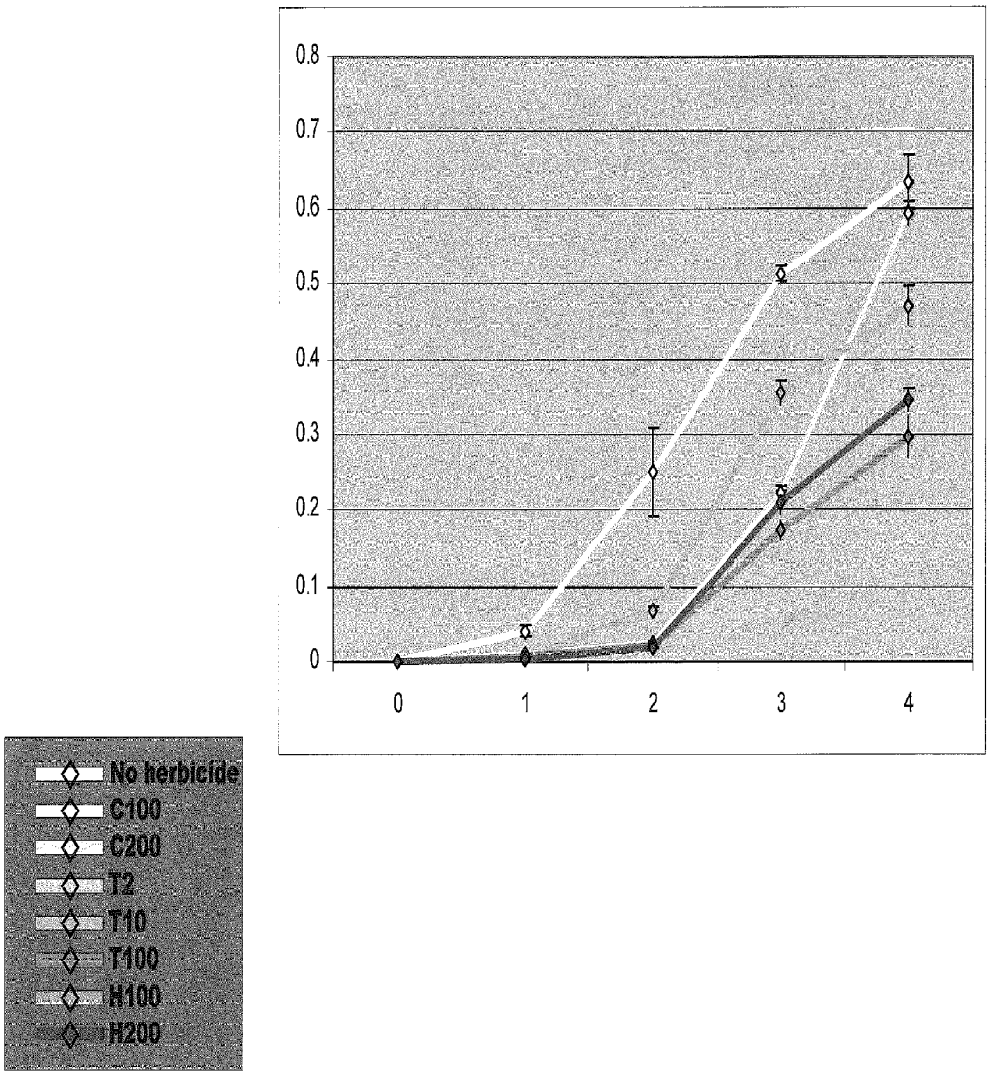


FIGURE 82

I1781L duplV2075

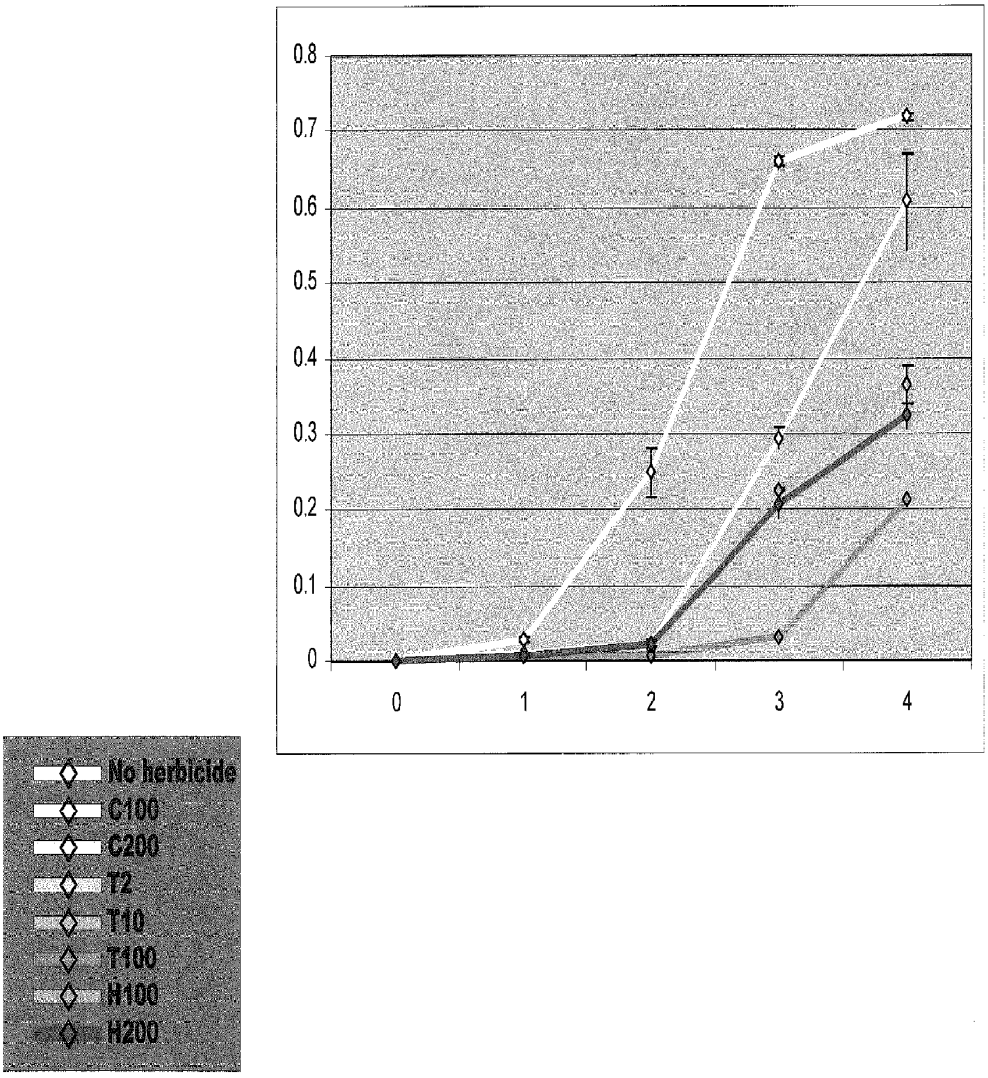


FIGURE 83

I1781L V1864F

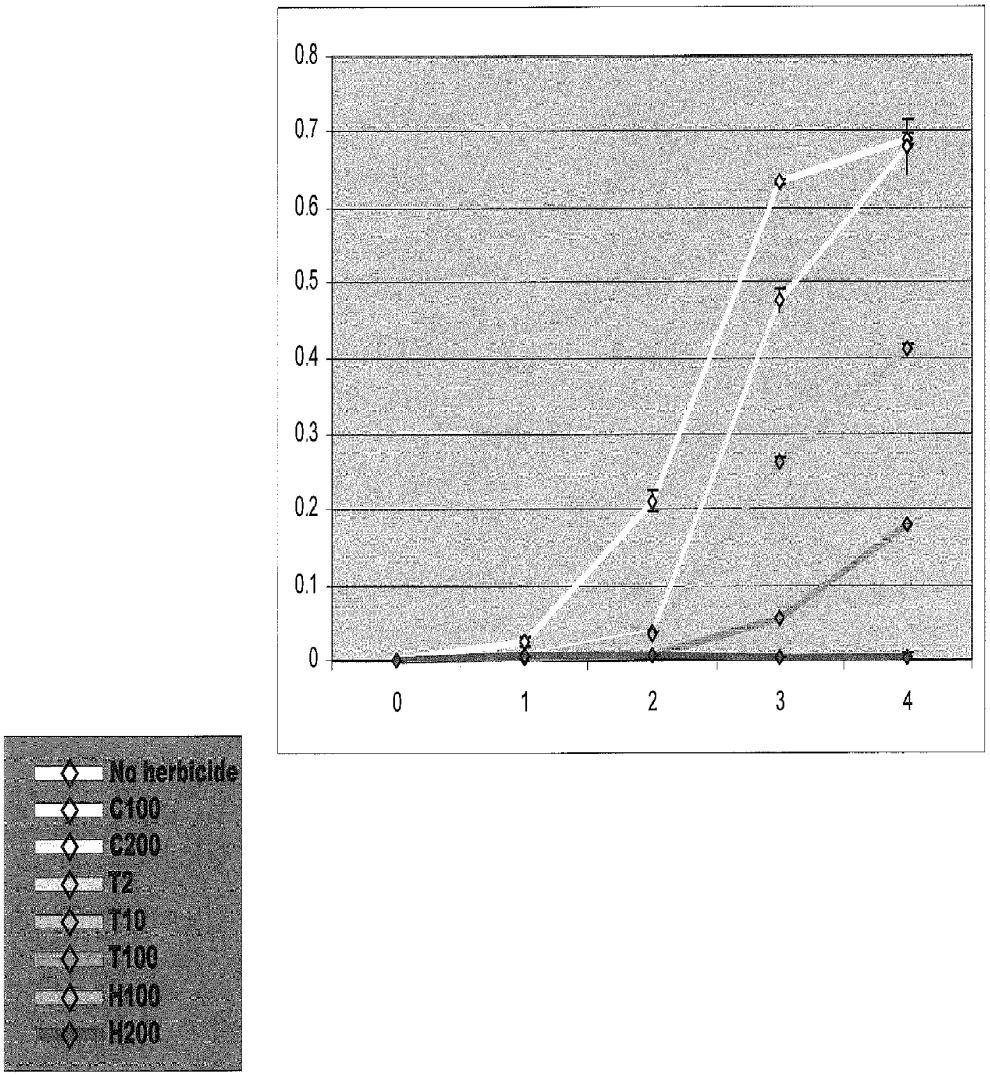


FIGURE 84

I1781L V2098G

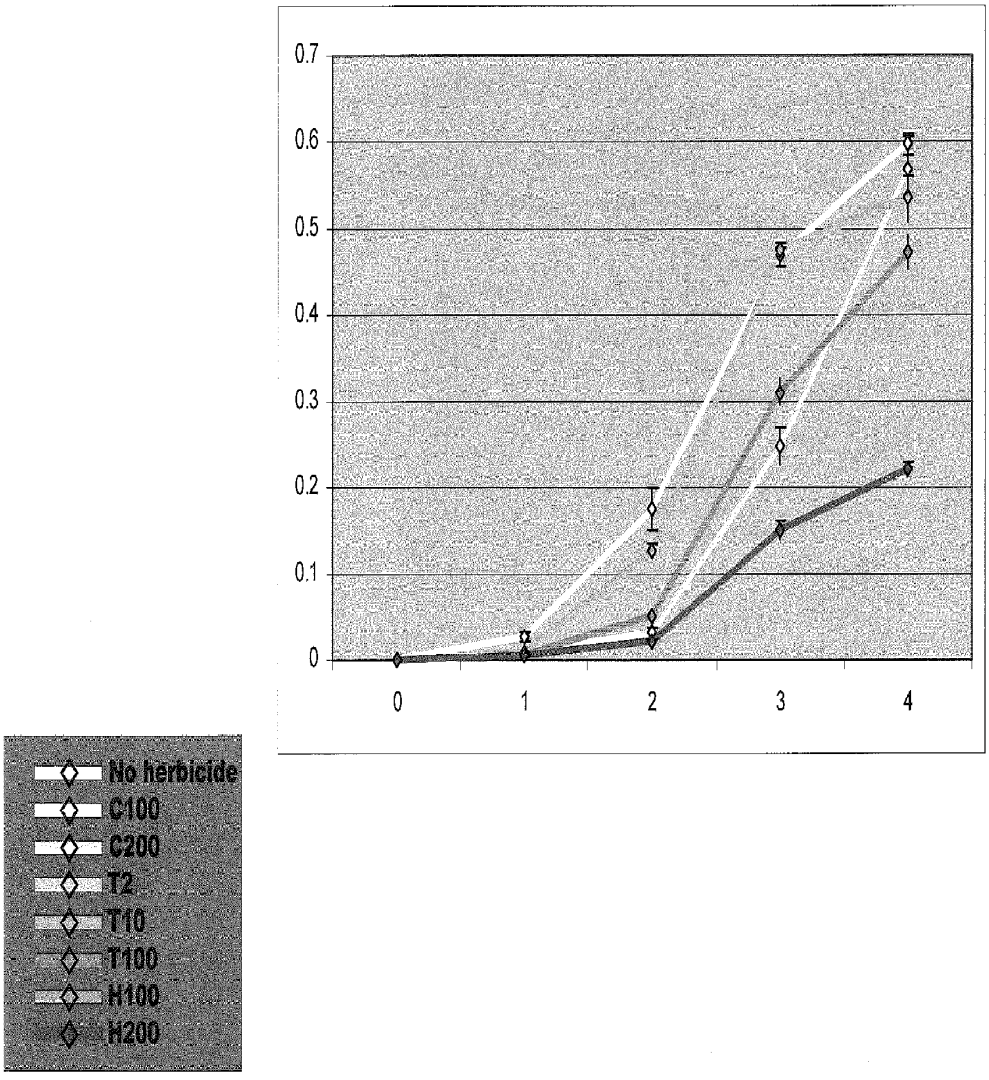


FIGURE 85

W1999G V2049I

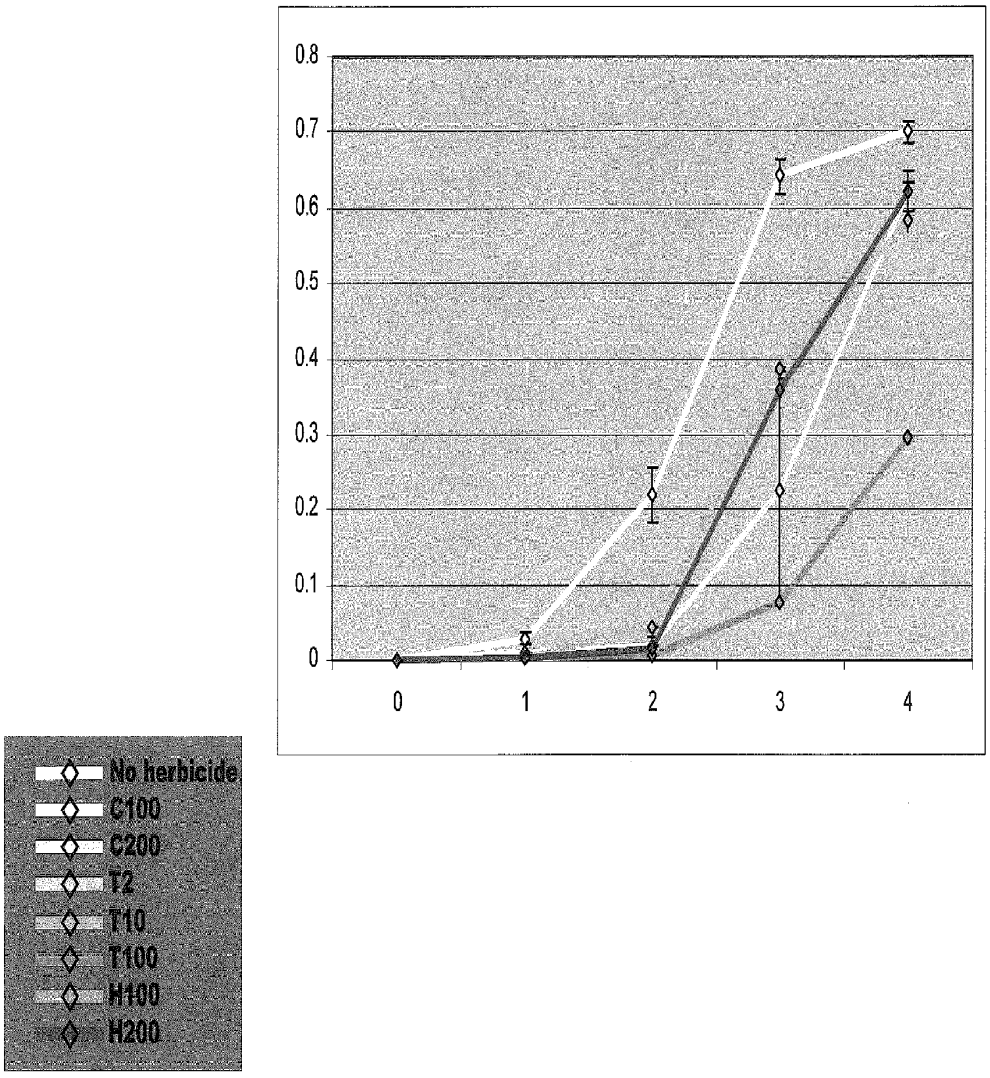


FIGURE 86

W1999G D2078G

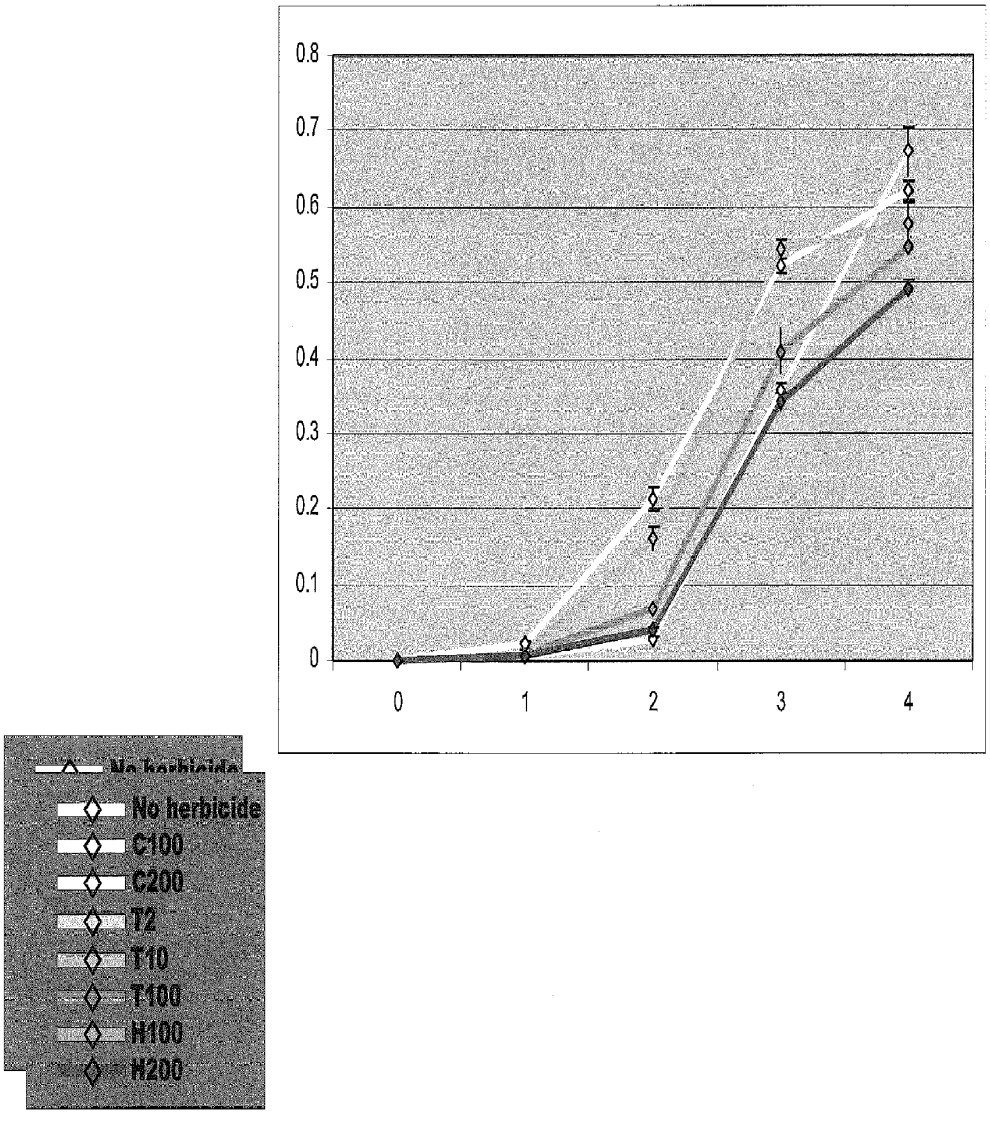


FIGURE 87

W1999G G2096A

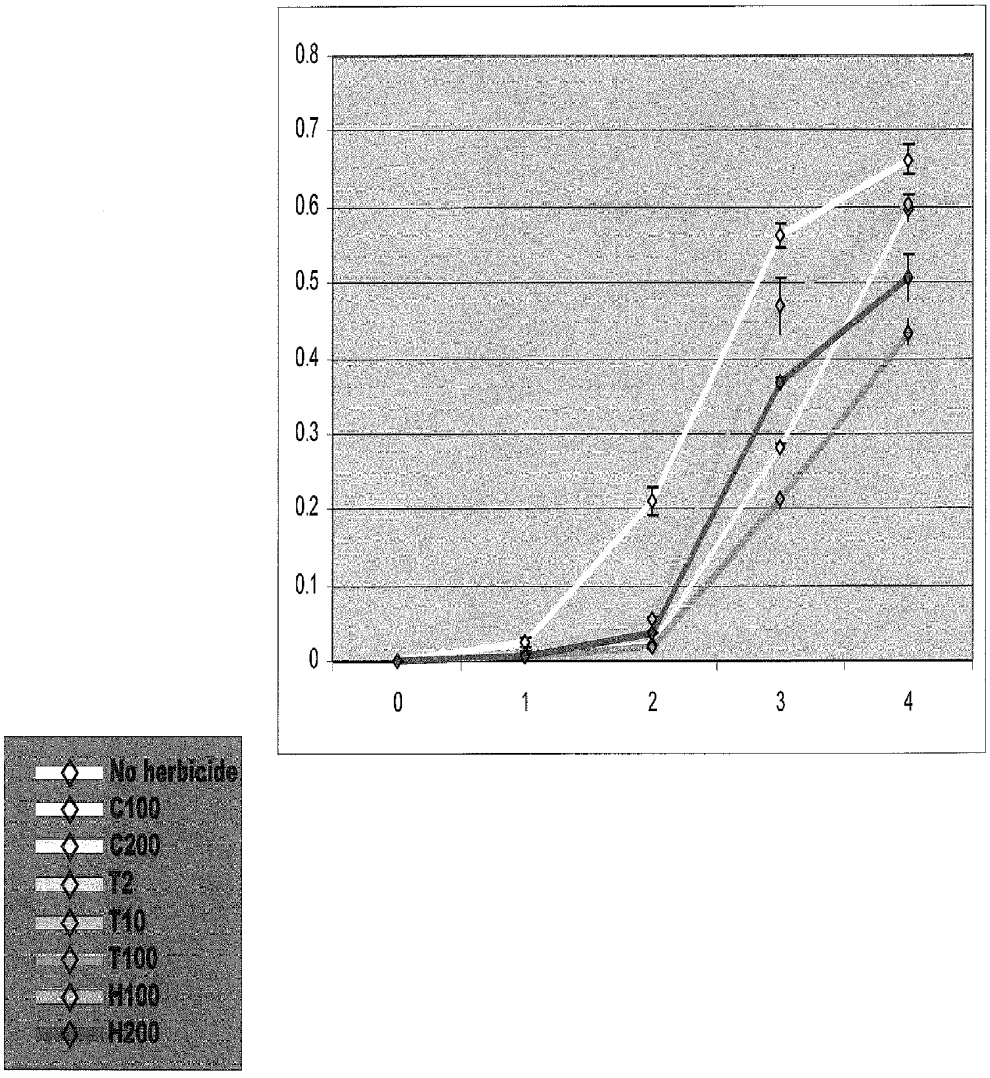


FIGURE 88

A1837V V2075I

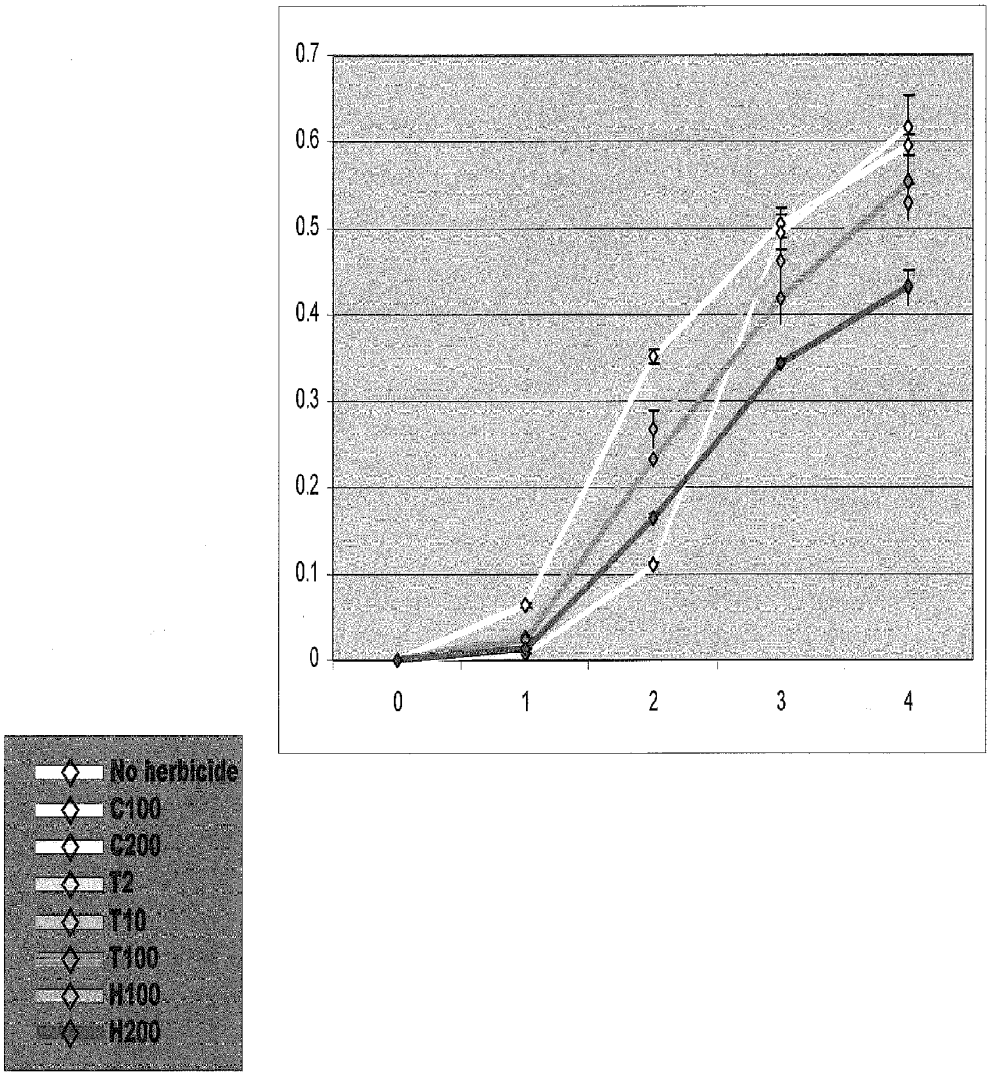


FIGURE 89

W1999C V2075I

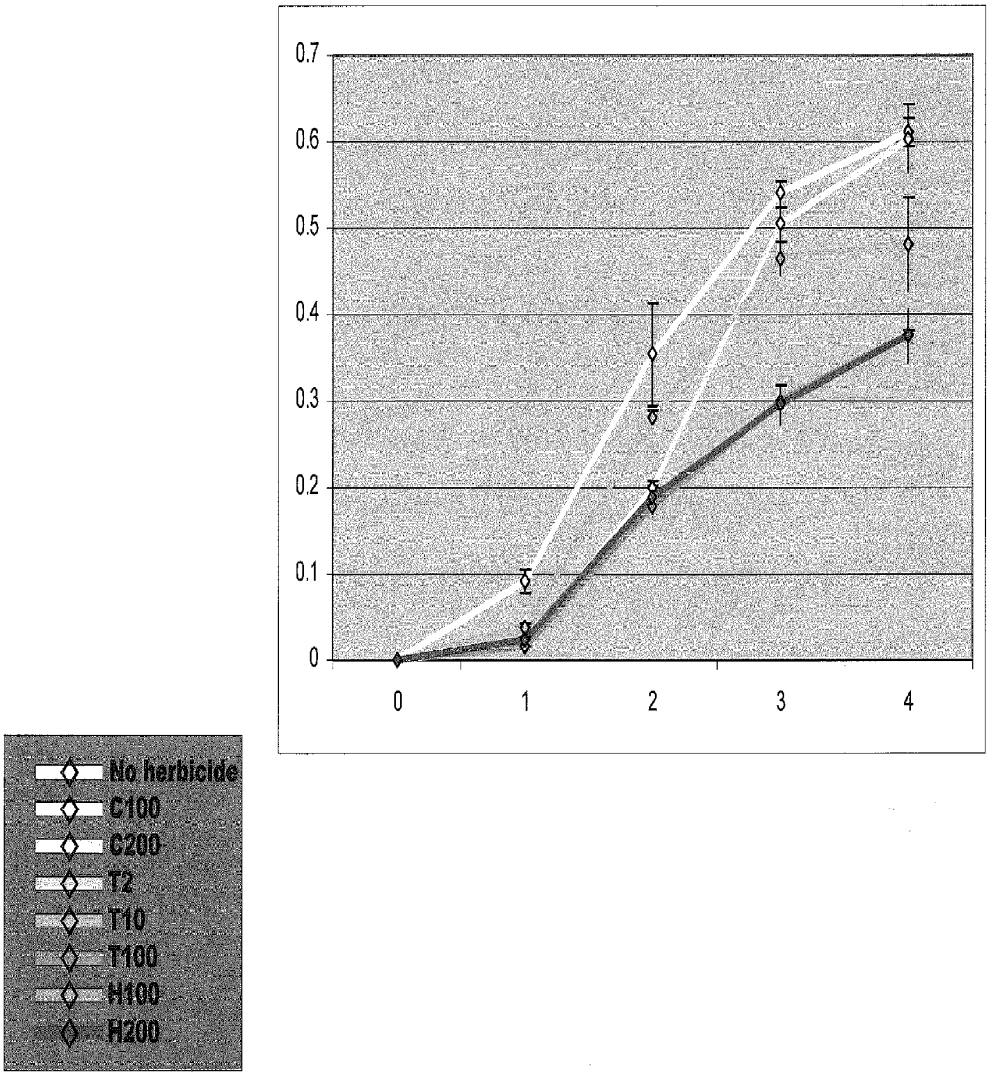
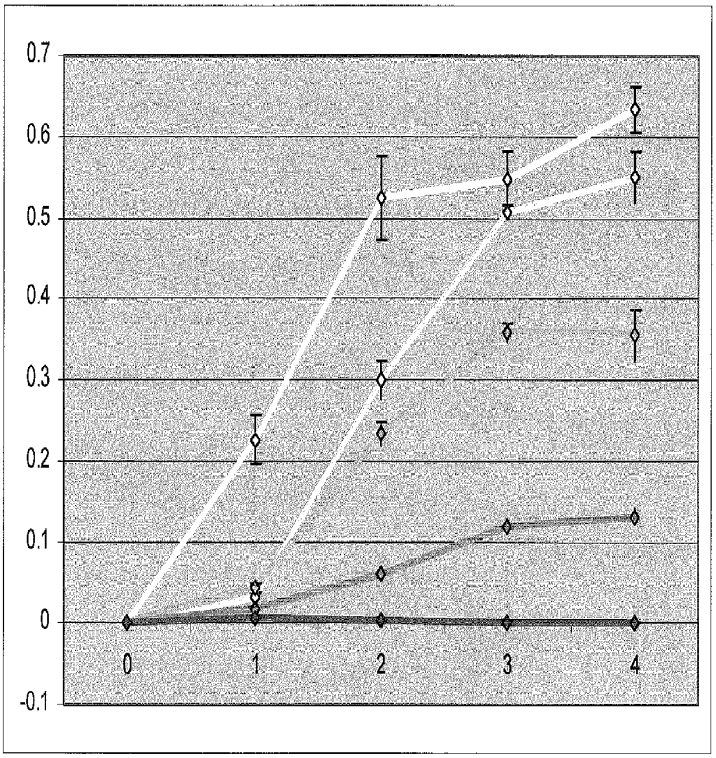


FIGURE 90

V2049C D2078G



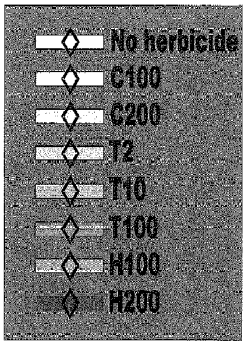


FIGURE 91

D2078G C2088W

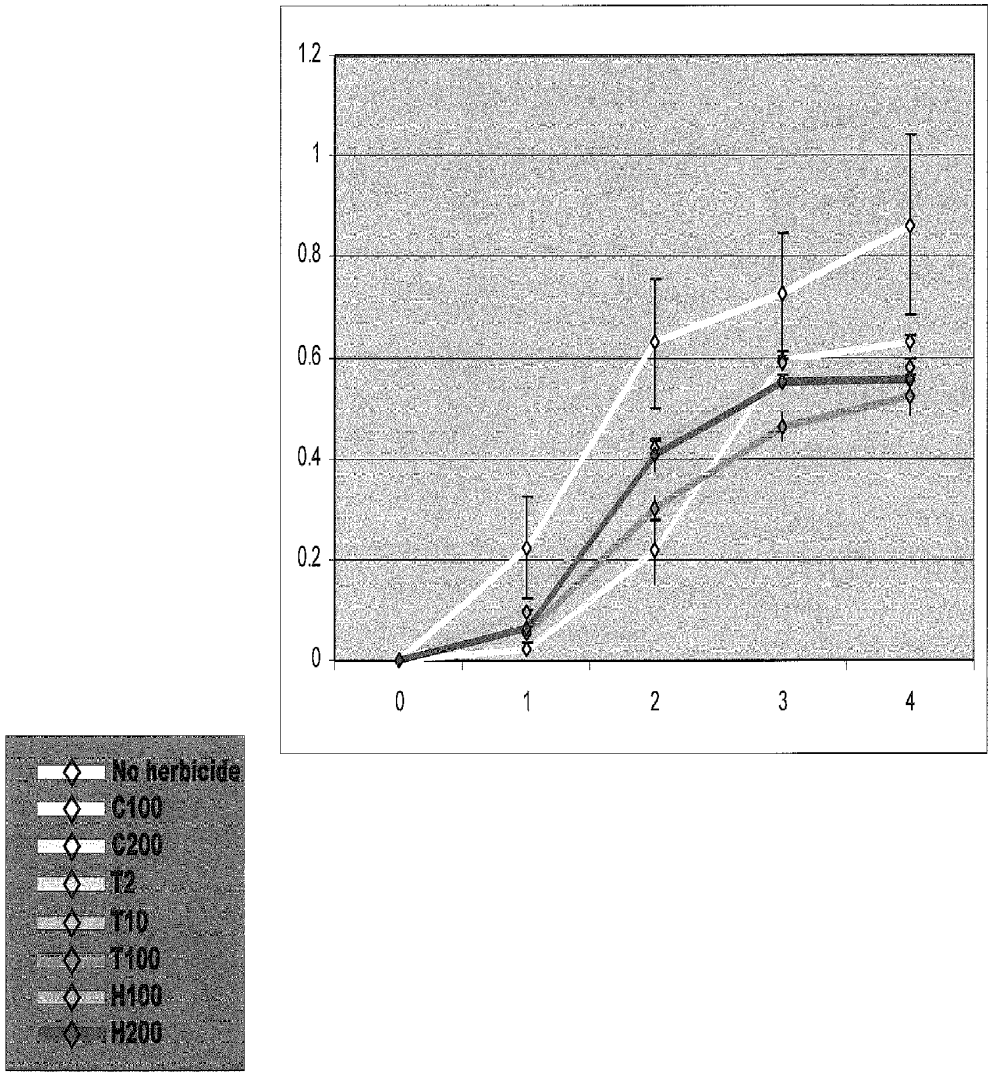


FIGURE 92

I1781L

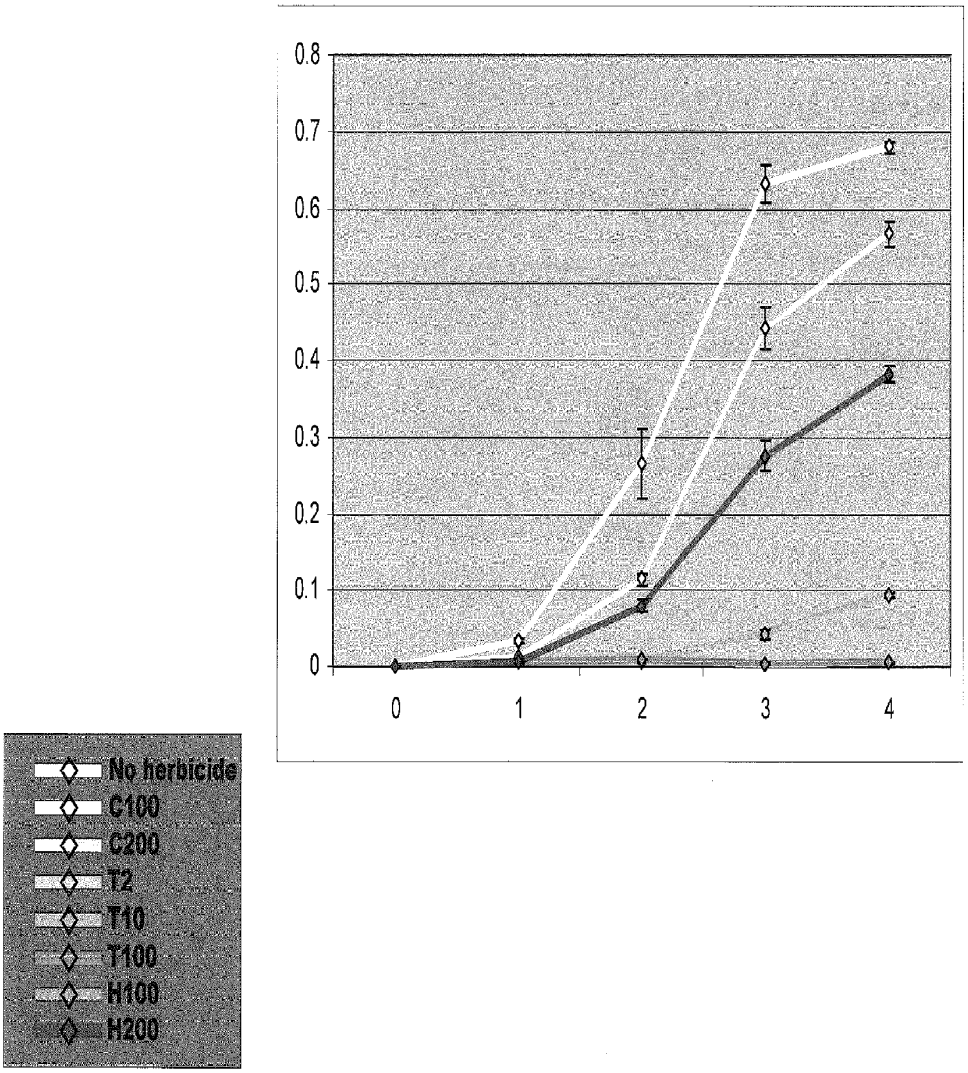
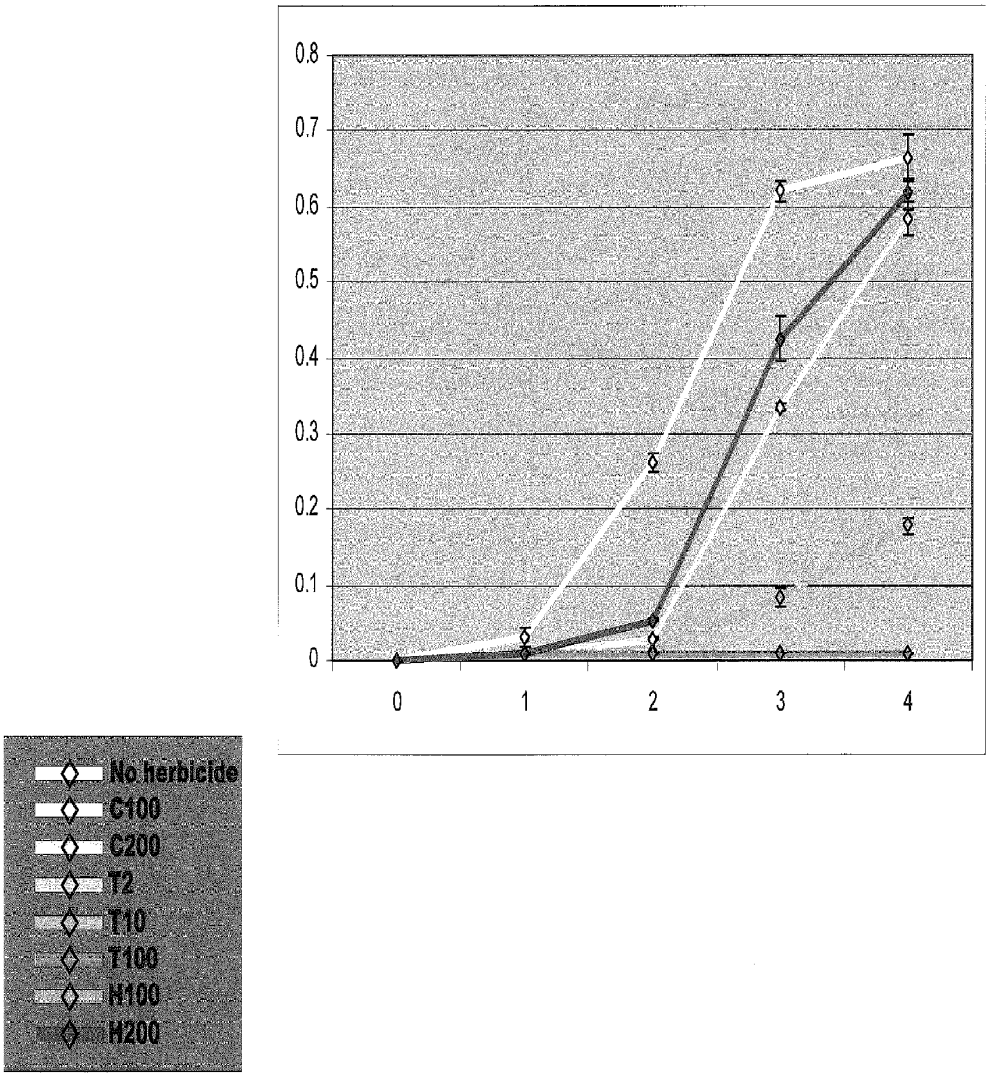


FIGURE 93

W1999G



Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	METHODS AND COMPOSITIONS FOR ISOLATING, IDENTIFYING AND CHARACTERIZING MONOCOT PLASTIDIC ACCASE HERBICIDE TOLERANT MUTATIONS USING A MODEL SYSTEM
First Named Inventor/Applicant Name:	Leon Neuteboom
Filer:	Lawrence Joseph Carroll
Attorney Docket Number:	B248 1400.P1

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Miscellaneous-Filing:				
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Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**560**

Electronic Acknowledgement Receipt

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Application Number:	61559618
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Confirmation Number:	3269
Title of Invention:	METHODS AND COMPOSITIONS FOR ISOLATING, IDENTIFYING AND CHARACTERIZING MONOCOT PLASTIDIC ACCASE HERBICIDE TOLERANT MUTATIONS USING A MODEL SYSTEM
First Named Inventor/Applicant Name:	Leon Neuteboom
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5	Drawings-other than black and white line drawings	Figures_54_93.pdf	5032988 b7d2d1ee424f6bfb5db137c1141a219de6ee32a1	no	40
Warnings:					
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Information:					
6	Fee Worksheet (SB06)	fee-info.pdf	31862 b069a39590f03158cde2ea4e2686fd7e236269bc	no	2
Warnings:					
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		Total Files Size (in bytes):	12598278		

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

DocCode - SCORE

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Application Number: 61559618 Document Date: 11/14/2011

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Form Revision Date: May 1, 2009