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Identification of a Mutant Resistant to Acetolactate Synthase Inhibitor Herbicides Through Mutagenesis in *Camelina sativa* (L.) Crantz

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ABSTRACT: Herbicide tolerance in crops is an effective way to manage weeds and maintain stable yield. *Camelina sativa* (L.) Crantz is commercially grown for the biofuel and bio-industrial markets. Having tolerance to widely used herbicide products can improve a variety's yield potential due to minimizing yield risks from weed competition. In this study, we tested the time of treatments with EMS and created an EMS-induced population from Camelina line 'Ames1043'. A mutant, namely Nfld001, was found in the field after spraying sulfonylurea (SU) herbicide Harmony™. The M_2 mutant and the M_3 population were tested by KASP assay and further confirmed on Chr09 by sequencing the ALS gene region covering the mutation. The mutation happened at the 197 amino acid with a transition from CCT to CTT causing the change of proline to leucine in the ALS protein. The mutant will be used for breeding camelina lines with better weed management and stable yield.

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INTRODUCTION

Efforts are expanding to cultivate Camelina (*Camelina sativa* (L.) Crantz as a low carbon intensity feedstock for biofuel production. Effective weed management is one of the primary obstacles to extensive commercial Camelina production. Since Camelina is frequently used as an "intermediate" crop which fits in close rotation with other crops, tolerance to carryover herbicides from previous crops is also needed.

Three methods are commonly utilized for weeding, including tillage, hand weeding, and the use of herbicides. Herbicide application is the most appropriate technique for effective weed management in large scale farming. So far, 19 herbicide groups with different sites of action have been described (chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://www.uidaho.edu/-/media/UIdaho-Responsive/Files/cals/centers/Kimberly/weed-science/2020-take-action-herbicide-classification-chart.pdf) and most have been applied in agriculture (*www.IWillTakeAction.com*). Group 2 herbicides inhibit the activity of the acetolactate synthase (ALS) enzyme in most plant species. There are five subgroups of Group 2; imidazolinone (IMI), pyrimidinylthiobenzoic acid, sulfonylaminocarbonyltriazolinone, sulfonylurea (SU), and triazolopyrimidine. SU and IMI are popular in global agriculture (Gutteridge et al., 2012).

Tolerance to Group 2 herbicides has been widely studied since mutations were first identified in the *ALS* gene in a weedy species in the 1990s. Table 1 lists the current known mutations and their earliest references. These mutations each show different degrees of tolerance to SU and IMI herbicides (Tranel et al., online 2024). During the past 30 years, numerous herbicide-tolerant weed and crop species have been identified or developed, including a tolerant Palmer amaranth (Singh et al., 2019), sunflower (Kolkman et al., 2004), Johnsongrass (Hernandez et al., 2015), sorghum (Werle et al., 2017), and canola (Hart 2018), etc. Resistant varieties have also been developed in soybean (Dupont Pioneer), canola (Cibus), camelina (Yield 10), and rice (Fartyal et al., 2018) etc.

Table 1. Mutations in the ALS genes among species

AA	Subs									References
Ala 122	Thr	Val	Tyr	Ser	Asn					Bernasconi et al., 1995
Pro 197	Thr	His	Arg	Leu	Gln	Aer	Ala	Ile	Ser	Saari et al., 1990

Ala 205 Asp	Val	Phe			Woodworth et al., 1996
376 Arg	Glu				Whaley et al., 2007
377 Trp	His				Massa et al., 2011
574 Ser	Leu	Gly	Met	Arg	Bernasconi et al., 1995 Mcnaughton et al.,
653 Gly	Thr	Asn	Ile		2001
654	Glu	Asp			Laplante et al., 2009

In camelina, cultivated lines are hexaploidy and have three copies of ALS genes in the genome. Mutations (Table 1) in one or more copies may cause changes in herbicide tolerance. Hanson (2010) found a single-point mutation (G to T) in the *als1* gene of the resistant biotype *Camelina microcarpa* which conferred a change from the amino acid tryptophan to leucine at position 572. Walsh (2012) subsequently found camelina mutants that had increased resistance to imazethapyr, sulfosulfuron, and flucarbazone, with the SM4 mutant showing the highest resistance. Hubert et al., (2018) registered the line, WA-HT1, with residue tolerance to imazamox (Beyond) herbicide. Residue herbicide has been a problem for the later crop in rotation if they are not tolerant to residue herbicides. Non-tolerant camelina is sensitive to ALS herbicides. Development of camelina lines with residue tolerance is very important for breeding. More recently, in 2021 Smart Earth Camelina (SEC) announced the world's first Group 2 herbicide resistant *Camelina sativa* variety 'New Gold' (https://www.smartearthcamelina.ca/blogs/press-releases/worlds-first-group-2-herbicide-resistant-camelina-seed-launches). However, detailed information of the mutations was not disclosed for the varieties developed. Lastly, Yield 10 Bioscience produced transgenic camelina lines tolerant to broad leaf herbicide application, as well as soil residues, of group 2 herbicides IMI and SU (https://www.yield10bio.com/blog/development-of-herbicide-tolerant-camelina-remains-on-track).

In order to create further mutations, a few methods can be applied. Chemical or physical mutation, transgenics, gene editing, TILLING, and RNAi are all potential routes for creating phenotype altering mutations in plants (Chaudhary et al., 2019). Among these methods, Ethyl methanesulfonate (EMS)-induced mutation has been widely used for its easiness, low cost, and higher efficiency (Chen et al., 2023). Walsh et al. 2012 Camelina seeds (cv. Cheyenne and Calena) were soaked overnight in 0.3% EMS in 0.1 M phosphate buffer, pH 7.5. Buchsenschutz-Nothdurft et al., (1998) tested the emergence rate of camelina lines after treated with 2.5 and 5% EMS for 2, 4 and 8 hours separately. Neumann et al., (2021) treated camelina seeds of Ames 1043 with 30 mM EMS for 17 h at room temperature using mild agitation. The M_3 mutation population was created for further analysis. According to the high mutation density observed (higher than 1/50 kb), they expected that 1000 families would carry at least a null mutation for the average gene and several missense mutations (Comai and Henikoff, 2006). For other species, the protocols are different in EMS concentration and time for treatment (Till et al., 20023; Xin et al., 2008). Further for the mutation rates, Till et al., (2003) have used the degree of embryo lethality assayed in the siliques of M1 plants as an indicator of mutation frequency. Because of the difference in time and concentration for the EMS treatment, it is necessary to have a protocol for the creation of camelina EMS populations.

In this study, we evaluated the time of EMS treatment and created a mutation population using EMS. The M_2 population was sprayed with SU herbicide. The survivors were screened by KASP assay with primers designed for beneficial *ALS* mutations. The mutation was confirmed by markers and sequencing. This confirmed mutation is now utilized in a camelina breeding program.

MATERIALS AND METHODS

An EMS-induced population was created from Ames1043 (Neumann et al., 2021) variety in 2022. Batches of 0.1 g seeds were treated with 0.4% of EMS for 2, 4, 6, 8, 10, and 12 hours in vials with gentle shaking. The seeds were then washed at low speed in beaker five times each for 5 mins with distilled water. The treated M_1 seeds were planted in flats in growth chamber controlled at 12 hours for day and 12 hours for night with a steady temperature of 23°C. Rates of survival were recorded. The surviving plants from 8, 10, and 12 hours were transferred to pots in the greenhouse. The seeds from all surviving M_1 plants were harvested and mixed into a bulk of 225 g M_2 seeds. The M_2 seeds were shipped to Nampa, Idaho and planted in the field in spring 2023. At the four-leaf stage, plants were sprayed with SU herbicide Harmony™ at the rate of 0.4 oz/ac. Surviving plants were identified and seed was harvested for further analysis.

Leaf samples from subsequent generations were collected in tubes and DNA was extracted using the 2 × CTAB method (Wang, 2007). For detection of the *ALS* mutations defined in Table 1, such as mutations 197, 205, 376, 377, 574 and 653, primers (Table 2) were designed using Batch Primer 3 (https://probes.pw.usda.gov/cgi-bin/batchprimer3). The selected *ALS* mutations were extracted

from the *ALS* gene database at International Survey of Herbicide Resistant Weeds (https://weedscience.org/Mutations/MutationDisplayAll.aspx). A KASP assay was used to screen the surviving plants on a QuantStudio™ - Real-Time PCR (Thermo Fisher Scientific, Waltham, MA). The PCR program included 10 mins denaturation at 94 °C, 10 cycles of touch down at the rate of 0.8 °C each cycle from 62 °C to 54°C; then 34 cycles of 94 °C for 20 s and 54 °C for 60 s for each cycle.

Table 2. Primers for the detection of *ALS* mutations

Name	Primer Sequence (5'-3")
	GAAGGTGACCAAGTTCATGCT
VBO12X	CATCAGTACCAATCATCCTACGAG
	GAAGGTCGGAGTCAACGGATT
VBO12Y	CATCAGTACCAATCATCCTACGAA
VBO12C	GTAGCGATCACGGGTCAAGT
VBO13X	GAAGGTGACCAAGTTCATGCT CGATCACGGGTCAAGTCC
VBO13Y	GAAGGTCGGAGTCAACGGATT GCGATCACGGGTCAAGTCT
VBO13C	CACCTCAACGATGGGAGTTT
VBO14X	GAAGGTGACCAAGTTCATGCT CGATCACGGGTCAAGTCC
VBO14Y	GAAGGTCGGAGTCAACGGATT CGATCACGGGTCAAGTCG
VBO14C	CACCTCAACGATGGGAGTTT
VBO15X	GAAGGTGACCAAGTTCATGCT CGATCACGGGTCAAGTCC
VBO15Y	GAAGGTCGGAGTCAACGGATT CGATCACGGGTCAAGTCA
VBO15C	CACCTCAACGATGGGAGTTT
VBO16X	GAAGGTGACCAAGTTCATGCT TTGGCATGGTTATGCAATG
VBO16Y	GAAGGTCGGAGTCAACGGATT CTTGGCATGGTTATGCAATT
VBO16C	TGTGAGCTCGGTTAGCTTTG
VBO16C-1	CCTCGCCGCTGGAA
VBO16C-2	TGTCACCCTCGCCG
VBO16C-3	TGTCACCCTCGCCGCTGG
VBO17X	GAAGGTGACCAAGTTCATGCT TTGGGGTGAGGTTTGATGAT
VBO17Y	GAAGGTCGGAGTCAACGGATT TTGGGGTGAGGTTTGATGAA
VBO17C	TTCGCCCTACTAGCAAAAGC
VBO18X	GAAGGTGACCAAGTTCATGCT CCTCAAGCTTACCCGTGACAC
VBO18Y	GAAGGTCGGAGTCAACGGATT CCTCAAGCTTACCCGTGACAT
VBO18C	TTGGGGTGAGGTTTGATGAT
VBO19F1	TCTCAACACAACAGCCAATGTCTCAA
VBO19F2	CCTCAACACAACAACCAATGTCTCAAC
VBO19F3	GCTCAACACAACCACCAATGTCTC

For amplicon sequencing, PCR was completed using the VBO19 primer pair to amplify all three camelina ALS gene copies. The PCR cycle includes 3 min at 94 °C, followed 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 2 min at 72 °C. Amplicons were purified using a QIAquick PCR Purification Kit (Cat. No./ ID: 28104) according to manufacturer recommendations. The purified amplicon was sent to Plasmidsaurus (Eugene, OR) for long nanopore sequencing. The sequence alignment and analysis were done using MEGA-X (Tamura et al., 2021).

RESULTS

Determination for time of EMS treatment

To determine optimal length of time for EMS treatment of the seeds, time tests were completed. From the treatments of 2, 4, and 6 hrs., the survival rates ranged from 100% to 91.8%, with the rate decreasing as time increased (Table 3; Fig. 1). Following the 8-hour treatment, which had a survival rate of 75.3%, rates dropped more rapidly, with 12 hours having a survival rate of only 22.9%. Because of the compensatory effect of the three genomes in camelina, checking mutation rates in surviving plants is challenging (Till et al., 2003). Therefore, survival rates were used as a guide to determine optimal treatment length. As 8 hours was the longest the seeds could withstand without the survival rates dropping rapidly, it was determined that anything longer than 8 hours led to sufficient mutation occurring within the genome. Thus, we moved forward with analyzing the plants from the 8-, 10-, and 12-hour

treatments. Altogether, this accounted for 95 plants, which were grown to maturity and produced 225 g of M_2 seed. After the M_2 seeds were planted in the field, around 70,000 plants germinated.

Table 3. Germination rates of the EMS treatments

Date	0 hrs.	2 hrs.	4 hrs.	6 hrs.	8 hrs.	10 hrs.	12 hrs.
12/10/2021		84	92	73	81	81	83
12/13/2021	26	73	85	39	28	21	5
12/15/2021		84	92	65	75	40	26
12/17/2021	26	84	92	73	75	45	28
12/20/2021	25	84	92	73	64	37	19
12/23/2021	25	84	92	71	62	27	19
12/28/2021	25	84	92	67	61	27	19
%	96.2	100	100	91.8	75.3	33.3	22.9

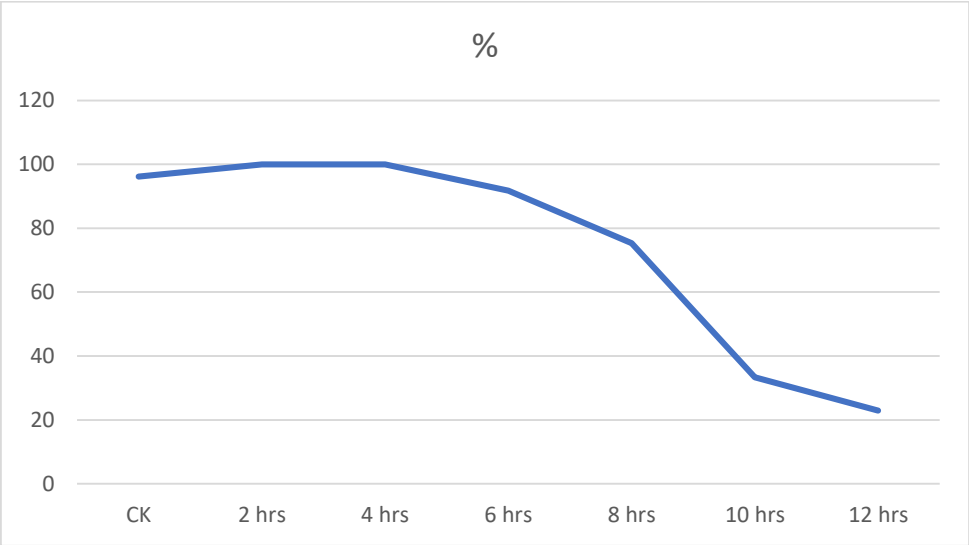

Fig.1 Germination rates with different time of EMS treatment

Identification of survivor plants

Five weeks after the field was sprayed with Harmony™, most plants started to die or show various symptoms of dying, such as yellowing, wilting, and/or drying. At this time, one survivor was identified with phenotypically normal growth (Fig.2). This plant was named Nfld001. Seeds of Nfld001 were harvested and planted in the greenhouse to generate an M_3 population. Between six to eight weeks after the spray, 20 additional surviving plants were identified from the field. Leaf samples were collected from these survivors and DNA was extracted for further analysis.

Fig. 2 Nfld001: The surviving plant in the field

Mutation screening by markers

To screen for mutations in the survivors, seven primer pairs were utilized in Nfld001. These included VBO12 to VBO18 (Table 2). Non-EMS treated Ames1043 was used as a control. Each sample had two replicates in the KASP assay (Fig.3). In the VBO12 assay, Nfld001 1; Fig.3). VBO12 tracks the P197L mutation (CCT to CTT), which is known to provide SU herbicide tolerance. For marker VBO16, showed a heterozygous mutant allele (allele 2) because VBO12 was designed to amplify the copy on Chr.04 and Chr.06. It was difficult to design primers amplifying only one copy of ALS. The controls showed only the homozygous wild-type allele (allele both Nfld001 and the control line showed the mutant allele (Fig.3). This mutation refers to W574L (TGG to TTG) and was shown to provide resistance to SU herbicides and IMI tolerance in some species (Bernasconi et al., 1995). Markers VBO13 to VBO17 did not show any difference between Nfld001 and the control line.

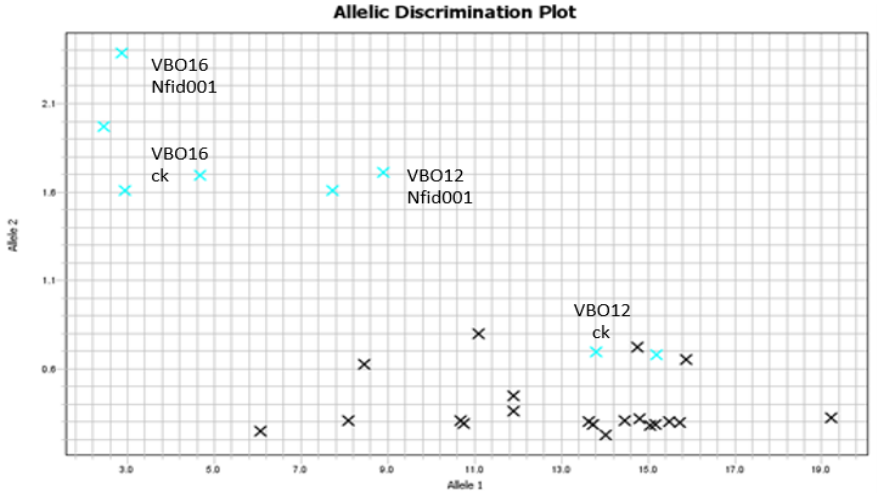

Fig. 3 Screening of Nfld001 by VBO primers

To screen the 20 additional survivors, the markers VBO12 to VBO18 were also used. No differences were observed between the survivors and the wild-type controls. Therefore, the data is not presented here.

Screening the M3 population

To confirm the marker results for the M_2 plant-Nfld001, VBO12 and VBO16 were used to screen the M_3 population (Fig. 4). VBO12 showed 106 plants were mutant for P197L mutation. Fourteen plants showed weaker signal for P197L mutant allele, but still within the range of mutant. VBO16 showed wild type signal for all M_3 plants along with the controls.

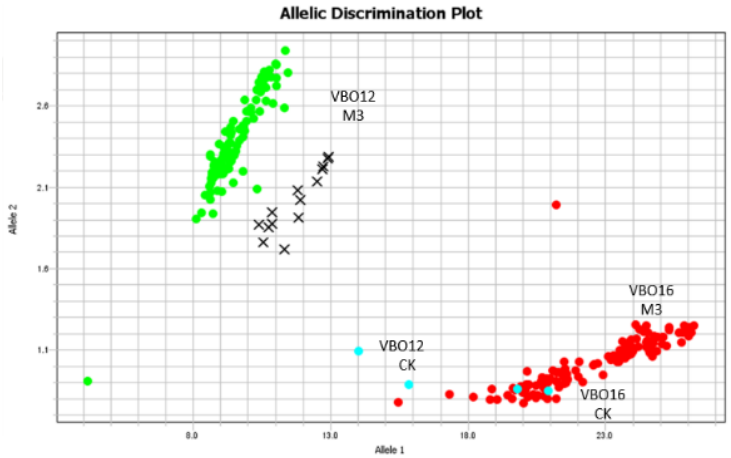

Fig.4 Screening of M_3 populations by VBO primers

Sequencing confirmation

To further confirm the mutations in M3 lines, PCR was used to amplify partial *ALS* gene sequences covering the mutations. The amplicons were then aligned against the sequences of each of the three *ALS* gene copies present in WT Ames1043 (Fig. 5). The alignments clearly showed a 197 mutation on Chr.09 ALS only. Specifically, a change of CCT to CTT was observed, substituting proline for leucine (P197L; Fig. 5).

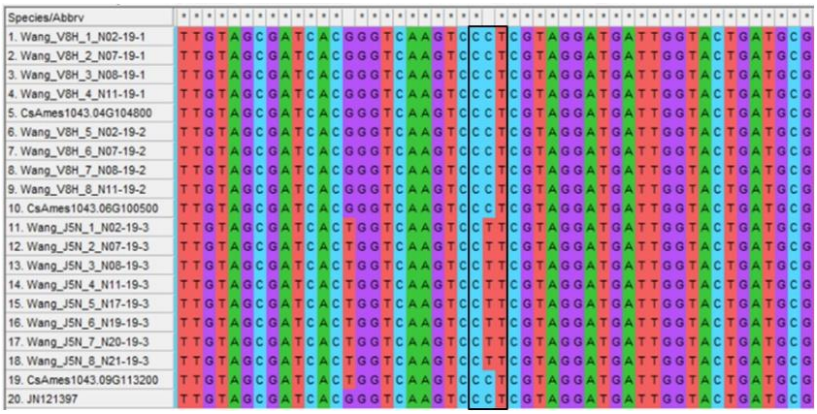

Fig.5 Sequence alignment of M_3 lines for the mutations

For marker VBO16, we checked the M_3 sequence at both the forward primer and common reverse primer regions. At the 574 amino acid location where the forward primes are in the genome sequence alignment, no mutation was found. In the common reverse primer region, there were a few mutations. Therefore, we can understand that VBO16 showing positive signals both for M_2 plant Nfld001 and the wild type controls was because of the non-specific amplifications. While for M_3 population, VBO16 can only amplify the wild type allele because there was no mutation at 574 of the amino acid sequence. The sequence data is not shown here.

DISCUSSION

Development of EMS mutation population

Based on the literatures, every publication used different concentration of EMS and treating time (Walsh et al. 2012; Buchsenschutz-Nothdurft et al., 1998; Neumann et al., 2021; Till et al., 20023; Xin et al., 2008). The concentration of EMS ranged from 0.3% to 5% and the time ranged from 2 hours to 17 hours. This was difficult for us to choose the right concentration and time to treat the seeds. To find the best concentration and time of treatment, we used the same rate 0.4% EMS to treat the seeds. At the fixed rate, we treated the seeds at 2, 4, 6, 8, 10 and 12 hours separately. We found that from 8 hours to 12 hours, the emergency rate dropped sharply with time increased. We supposed that the higher the death rate, the more mutations will happen. Till et al., (2003) have used the degree of embryo lethality assayed in the siliques of M_1 plants as an indicator of mutation frequency. However, camelina has three genomes. Their three copies of genes can compensate each other. Therefore, the M_1 plants of camelina either died because of too many mutations in the genome or were alive with good seed setting. It was true that all the M_1 plants survived set good seeds. We could not assess the mutation rate using the embryo lethality. We did not have the sequence to evaluate it either. However, the death rate of M_1 plants would be a good sign of mutation. For 8 hours and longer, the death rates went up sharpy, indicating an increase in mutations. Therefore, we decided to grow the 95 M_1 plants from 8 to 12 hours to M_2. We successfully obtained one true mutation P197L from the field, indicating that the treatment of EMS at 0.4% for 8 hours and over was effective to produce the mutations required. For the next, we will produce a larger M_1 population and continue the selection of other mutations.

SU mutations are important for herbicide resistance.

To date, *ALS* mutations have been found in different species (Table 1) which confer tolerance to SU or IMI herbicides, particularly at the 197, 205, and 574 amino acid locations (Saari et al., 1990; Woodworth et al., 1996; Bernasconi et al., 1995). Hubert et al., (2018) registered WA-HT1with residue tolerance to IMI herbicide because of a mutation at different position in genome than mutation 197, 205 and 574 etc. There were no published mutations for 197 or 574 in camelina. Therefore, the creation of these mutations in camelina is an important and necessary discovery for weed management. In this study, we successfully created the P197L mutation, introducing effective SU tolerance for farmers to efficiently manage weeds in the field and maintain stable yield of seeds. With the new traits for herbicide tolerance, camelina lines will increase the potential to be a good biofuel feedstock in the future. The P197L mutant achieves one of the overarching goals of our breeding program.

While the mutation showed resistance to SU herbicides, no resistance to IMI was observed due to no IMI treatment in the selection nursery. We did, however, test the M_3 population for IMI tolerance. Unfortunately, the P197L mutation was not tolerant to IMI herbicide (Data not shown). This was the first report in camelina. We need to develop other mutants that have both SU and IMI tolerance. It also highlights an area for future exploration, along with other herbicide traits such as HDDP-inhibitor tolerance. A mutant with only SU tolerance is not fully sufficient for breeding tolerant camelina varieties with tolerance to multiple groups of herbicides. Introducing novel traits and stacking them into camelina will greatly help with stable, economical production of camelina oil and weed management.

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