

Role of Genetic Engineering in Sunflower (*Helianthus annuus* L) Improvement and Current Status of Sunflower Research in Ethiopian: An Overview

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Abstract: Sunflower is one of the four most important oilseeds in the world. Because of its high quality of oil about 90% of the total production of oil is used for human consumption. Although sunflower is not widely grown in Ethiopia, the country has immense potential for its production. Sunflower research was started in the late 1960s with slow laps compared to other cereal crops. Obviously, Biotechnology can speed up plant breeding schemes, with many of the techniques complementing rather than substituting conventional methods and some biotechnological products have had a strong impact upon production systems because they have also facilitated crop management. Various approaches in the production of transgenic plants have been used, but with low efficiency in transformation; direct and indirect methods. Globally, the role of genetic engineering in the improvement of sunflower have done a big effort to combat biotic and abiotic factors. A multitude of factors such as insects and diseases reduce the sunflower yield and molecular biology research with a focus on transgenic sunflowers in different aspects. In an era marked by political and societal pressure to reduce the use of pesticides, crop protection by genetic improvement provides a promising alternative with no obvious impact on human health or the environment. Genome editing is one of the genetic levers that can be adopted technology in agriculture. CRISPR technology now a day gives a solution for different production constraints of different crops including sunflower. Traditional sunflower oil has been the major polyunsaturated oil used for many years in human nutrition to replace saturated fat, in an attempt to reduce cardiovascular diseases. Different GMO of sunflower has been developed with increased oil quality and quantity as well. Generally, sunflower research in Ethiopia has to plan on the development of sunflower varieties using modern tools of biotechnology especially CRISPR/Cas9 in the future in order to combat the dynamic environmental conditions and to hasten the existing sunflower breeding effort for the betterment of future generations as well. Therefore, reviewing the current status of sunflower research in Ethiopia and the role of genetic engineering in sunflower improvement globally is the main objective of this paper.

Keywords: Biotechnology, CRISPR/Cas9, Genome-editing, GMO, genetic-engineering, sunflower

1. INTRODUCTION

Sunflower is one of the major sources of edible oil in the world and assumed to be originated from southern United States where its progenitor *H. annuus* was found (Heiser *et al.*, 1969; Putt, 1997; Harter *et al.*, 2004; Yönet and Yilancioglu, 2018). Sunflower is a wide spread oilseed crop of the world and it is almost grown in all continents (Damodaran and Hege, 2007&2010). Evidences suggested that sunflower was introduced to the North Horn of Africa including Ethiopia by the Italians more than 160 years ago (Getinet and Nigussie, 1992). Although sunflower is not widely grown in Ethiopia, the country has immense potential for sunflower production. Even though, Sunflower production is low

compared to other oil seeds in the country. Currently, inauguration of recent edible oil private factories has changed the scenario on growing sunflower due to high demand for raw material.

Ethiopia is making huge improvement in agriculture sector but still the gap exists in terms of extreme shortage of edible oil. During 2017 production year, Ethiopia satisfied 4% its requirement of edible oil by domestic oil and 96% by imported oil. Both conventional and non-conventional oilseed crops are important to fill the gap between demand and production (Popescu, 2018). With increasing demand, limited domestic production and the country's heavy reliance on imported palm oil, there are frequent supply shortages especially in urban areas. In addition, as some consumers become increasingly diet conscious, they are looking for healthier alternatives to palm oil. There is an increasing preference towards alternative edible oils containing saturated oils and fats. Most Ethiopian consumers prefer sunflower, Niger seed, and soybean oils as healthier. Due to these changes in consumer preferences, consumption of sunflower oil has almost tripled over the last couple of years and that of palm oil has dropped considerably (<https://www.ministryoftrade-et.com>). Different privately owned edible oil factories established and foresee producing mainly sunflower and soybean oils. This will induce more production of oilseeds locally (soybeans, sunflower, and Niger seed) and even create some opportunities for imports.

Obviously, new tools of biotechnology can speed up plant breeding, with many of the techniques complementing rather than substituting conventional methods (Abah *et al.*, 2010) and some biotechnological products have had a strong impact upon production systems because they have also facilitated crop management. This has led to a major increase in the total area devoted to genetically modified (GM) maize, soybean, cotton, and rapeseed production, which now exceeds 90 million ha, most of which are distributed among the 14 countries in which these crops have been authorized (James, 2004). Various approaches in production of transgenic plants have been used, but with low efficiencies in transformation: Polyethylene glycol (PEG) (Moyne *et al.*, 1988), microprojectile bombardment (Knittel *et al.*, 1994; Laparra *et al.*, 1995; Hunold *et al.*, 1995) and electroporation (Kirches *et al.*, 1991). Recent techniques in genetic engineering widely used is CRISPR technology. The CRISPR gene-editing technology is composed of an endonuclease protein whose DNA-targeting specificity and cutting activity can be programmed by a short guide RNA. CRISPR stands for Clustered Regularly Inter-Spaced Short Palindromic Repeat DNA sequences. Although the name CRISPR was coined much later (Jansen *et al.*, 2002). Therefore, reviewing the role of genetic engineering in sunflower improvement globally and the current status of sunflower research in Ethiopia is the main objective of this paper.

Role of Genetic Engineering in Sunflower Improvement: Globally

Domestication of sunflower by human for a particular desired character in a good environmental condition increases evolution in different characters. But the cost of reduced defense mechanisms against biotic and abiotic factors also increases. Studies shows that Sunflower growth characters under good environmental condition is greater for the domesticated genotype population than that for the wild population. While, with a drop in defense response in domesticated sunflowers when exposed to biotic and abiotic stresses. Additionally, it was found that lepidopteran pests preferred domesticated sunflowers than wild sunflowers. It was also revealed that *Botrytis cinerea* and drought had more negative consequences on domesticated sunflowers than the native plants. A multitude of factors such as insects and diseases reduce the sunflower yield.

Molecular biology research with a focus on transgenic sunflowers to develop pest resistance, herbicide resistance, increasing oil yield of sunflower holds indispensable as well as its study on ecological impact is pressing (Chen and Welter, 2002; Mayrose *et al.*, 2011; Malnoy *et al.*, 2016; Wang *et al.*, 2017). Many Transformants carry valuable characters such as: tolerance to herbicides, agronomic performance, resistance to insects, virus, bacterial, nematode and pathogens, quality traits (protein, oil, carbohydrate and fatty acids including amino acids composition), modified reproductive capacity, photosynthetic, enhancement and yield increase, delayed senescence, enhanced flavor and texture, longer shelf life, and more healthful produce (Dunwell, 2000). The production of GMO has been revolutionized for the last decade by private and public institutions. Monsanto Company, has played a major role in applying for the maximum role followed by Dupont-Pioneer, Syngenta (Gill and Friebe, 1998; Sybenga, 2012; Sharma *et al.*, 2012; Singh, 2016).

Techniques in Genetic Engineering

Biotechnology can speed up plant breeding scheme, with many of the techniques complementing rather than substituting conventional methods (FAO, 2005) and some biotechnological products have had a strong impact upon production

systems because they have also facilitated crop management practices as well. This has led to a major increase in the total area devoted to genetically modified (GM) maize, soybean, cotton, and rapeseed production, which now exceeds 90 million ha, most of which are distributed among the 14 countries in which these crops have been authorized (James, 2004). Different approaches in production of transgenic plants have been used, but with low efficiencies in transformation: Polyethylene glycol (PEG)-induced vector uptake of pCAMVNEO into protoplasts isolated from sunflower seedling hypocotyls (Moyné *et al.*, 1988), microprojectile bombardment (Knittel *et al.*, 1994; Laparra *et al.*, 1995; Hunold *et al.*, 1995) and electroporation (Kirches *et al.*, 1991). PEG-induced vector uptake turned out to be mainly labor intensive and some other protocols with *Agrobacterium*-mediated transformation of sunflower plants have been used (Bidney *et al.*, 1992; Laparra *et al.*, 1995; Rao *et al.*, 1999; Weber *et al.*, 2003; Ikeda *et al.*, 2005; Mohamed *et al.*, 2006). An emerging and recent techniques in genetic engineering widely used is CRISPR technology.

The Rise of CRISPR as the Genome-Editing Technology

DNA is the instruction manual for life on Earth. It encodes the fundamental properties of an organism; how it lives, grows and reproduces. Changing a DNA sequence in a living cell is known as genome editing or gene editing. For a long time, this was either impossible or extremely challenging. The discovery of CRISPR genome editing has made this process much easier. In 2012, research by IGI founder Jennifer Doudna, Emmanuelle Charpentier, and their teams developed a method of re-purposing a bacterial immune system called CRISPR to make breaks in DNA at precise locations, using a CRISPR-associated enzyme (*Cas9* protein) like molecular scissors to cut DNA. Scientists can now edit the genome of living organisms by adding new fragments of DNA for the cell to use as a template when it repairs the break in the DNA. In this way, scientists can replace a disease-causing mutation with a healthy sequence or make other modifications to the genome. Alternatively, scientists can use this method to “knock out” a gene entirely. A technique that is frequently used to study the functions of genes or to modify portions of the genome that affect how genes are expressed, known as “epigenetic editing”. Together, these methods give scientists powerful new tools to treat disease, improve agriculture, and study fundamental questions of biology.

Although the discovery of artificially designed mega-nucleases followed by ZFNs and TALENs successively increased the genome-editing efficacy, targeting different sites in the genome required re-design or re-engineering of a new set of proteins. The difficulty in cloning and protein engineering ZFNs and TALENs partially prevented these tools from being broadly adopted by the scientific community. In this respect, CRISPR has revolutionized the field because it is as robust as, if not more so than, the existing tools in terms of editing efficiency. More importantly, it is much simpler and more flexible to use (Adli, 2018). The CRISPR gene-editing technology is composed of an endonuclease protein whose DNA-targeting specificity and cutting activity can be programmed by a short guide RNA. Notably, CRISPR had been simply known as a peculiar prokaryotic DNA repeat element for several decades before it was recognized as the bacterial immune system and subsequently harnessed as a powerful re-programmable gene-targeting tool (Mojica *et al.*, 2000; Jansen, *et al.* 2002; Adli, 2018 and Bao *et al.*, 2019).

Basically, CRISPR stands for Clustered Regularly Inter-Spaced Short Palindromic Repeat DNA sequences. Although the name CRISPR was coined much later (Jansen *et al.*, 2002), these repeat elements were initially noticed in *Escherichia coli* by Dr. Nakata’s group (Ishino *et al.*, 1987). Interestingly, unlike typical tandem repeats in the genome, the CRISPR repeat clusters were separated by non-repeating DNA sequences called spacers. It took more than a decade for researchers to recognize the nature and origin of these spacer sequences. During the human genome project (HGP), the genomes of many other organisms, including many different phages, were also sequenced. The computational analysis of these genomic sequences led researchers to notice key features of CRISPR repeat and spacer elements. Firstly, the CRISPR sequences are present in more than 40% of sequenced bacteria and 90% of archaea (Mojica *et al.*, 2000).

A CRISPR/Cas9 model of Sunflower Resistance for Biotic and Abiotic Stresses

Breeders encounter yield losses, which can reach up to 100%, due to different biotic and abiotic stresses in different regions of the world each year. Downy mildew, broom rape, stem canker and head rot, and drought, salinity, heat and cold can be given as the examples for biotic and abiotic stresses, respectively. A gene editing model for sunflower resistances is constructed as: determination of the suitable genetic changes to perform and design of relevant plasmids; determination of plasmid transfer method into the sunflower genome and control for whether the plasmid is transferred and for whether gene editing is effective (Yönet and Yilancioglu, 2018). In sunflower, the release of genetically modified organisms

(GMO) must be carefully considered because of the agro-ecological implications of a possible transgene escape. Since it is an open-pollinated crop native to North America (Heiser, 1978; Harter *et al.*, 2005, Yönet and Yilancioglu, 2018) which has now wild relatives throughout the world (Faure *et al.*, 2002; Dry and Burdon, 1986; Quagliaro *et al.*, 2001; Ribeiro *et al.*, 2001; Mojica *et al.*, 2000; Poverene *et al.*, 2009). Crop-wild gene exchange allows transgene escape via gene flow in sunflowers (Whitton *et al.*, 1997; Linder *et al.*, 1998; Rieseberg *et al.*, 1999; Burke *et al.*, 2002). Transgenes from the crop could potentially disperse into wild or weedy populations enhancing their fitness and modifying their ecological interactions (Bao *et al.*, 2019; Burke and Rieseberg, 2003; Mojica *et al.*, 2000; Snow *et al.*, 2003; Mazhar *et al.*, 2021).

2. DEVELOPMENT OF BIOTIC STRESS TOLERANT TRANSGENIC SUNFLOWER

Disease Resistance Development Efforts

Sunflower production mainly affected, among other factors, by the presence of diseases caused by viruses, bacteria, fungi and nematodes. Sunflower hosts over 30 phyto-pathogenic disease-causing microorganisms, mostly fungi, which may, depending on climatic conditions that favor the occurrence of pathogens and the infective process, lead to a significant reduction on yield and quality of product (Zimmer & Hoes, 1978; Gulya *et al.*, 1997). Major Sunflower diseases are: Downy mildew, Phoma black stem, *Alternaria* Leaf Spot, Phomopsis stem canker, rust, Sclerotinia wilt, Sunflower mosaic virus, Verticillium wilt, Powdery mildew, head rot and Charcoal rot disease causes losses on more than 500 cultivated and wild plant species (Khan, 2007; Yönet and Yilancioglu, 2018). Some diseases have significant importance, like *Alternaria* leaf spot and Sclerotinia wilt and head rot, which are the most severe (Embrapa, 1983). *Alternaria* blight reduces seed and oil yield by 27-80% and 17-33% respectively (Mukhtar, 2009, Yönet and Yilancioglu, 2018). *Sclerotinia* has been reported to cause damage up to 50% in sunflower in UK (Tu, 1989; Burke and Rieseberg, 2003). The fungal Downy mildew can lead to more than 50% yield loss (Hvarleva *et al.*, 2009; Markell *et al.*, 2015). Sunflower species of *Helianthus* as wild *H. annuus*, *H. hirsutus*, *H. rigidus* and *H. tuberosus*, show resistance to *A. helianthi* (Lipps & Herr, 1986; Davet *et al.*, 1991). The interspecific hybridization may allow the incorporation of resistance genes in cultivated genotypes (Davet *et al.*, 1991). Some agronomic recommendation, planting dates and chemical control options, are in practices for the management of *Alternaria* disease.

Now a days; *Oxo*-transgenic sunflower plants were obtained by introducing wheat germin gf 2.8 *OXO* gene to confer resistance against fungal disease *Sclerotinia* head rot (Lane *et al.*, 1991; Lu *et al.*, 2000; Lu, 2003; Hu *et al.*, 2003). However, it has been of concern if *OXO* enzyme could be a human allergen (Jensen-Jarolim *et al.*, 2002). The probability of transgenic wild plants being a worse weed is scarce as *OXO* transgene will diffuse neutrally on its escape because the transgenic wild plants do not produce ample number of seeds than the wild population (Burke and Rieseberg, 2003). Human lysozyme gene under *CaMV 35S* promoter and *Nos* terminator in a binary vector containing *NPTII* and *GUS* marker genes was incorporated in sunflowers using hypocotyl explants with *Agrobacterium* mediated transformation conferred resistance against mold disease causing *Sclerotinia sclerotiorum* (Sawahel and Hagan, 2006). Genetic resistance to Sclerotinia wilt and head rot has been studied in several countries. Efforts have been made in breeding programs around the world aimed at finding resistance to the pathogen, but little progress has been made (Zimmer & Hoes, 1978; Gulya *et al.*, 1997). According to Chapman and Burke (2006) stated the possibility of “gene flow” concluding that the natural selection is the key in spread of favorable transgene alleles.

Insect Tolerance Development Efforts

On Sunflower, insects cause different type of problem. At the center of origin of sunflower, in North America, there are almost 50 species belonging to genus *Helianthus*. Almost 40% of at least 25 different insect species that constitute plagues for this crop are restricted to this genus. On the other hand, in Europe and South America most of the insects that affect sunflower are non-selective (Charlet *et al.*, 2009). And of 16 insect pests reported during 2001-2005 years, three are restricted to the genus *Helianthus* spp., being found only in the center of origin. The others are polyphagous and have a number of unspecific controllers, with the main cosmopolitan one being *Helicoverpa armigera* (Soyars *et al.*, 2018). The most frequent methods used for insect control in sunflower involve the use of pesticides. A number of chemical products are recommended to control insects that reduce crop stand.

Scientists observed that the transgenic plants in a greenhouse experiment even without the insect pests produced no difference in the seeds or inflorescences. The study concluded that the transgene itself didn't actually cause the benefit in these transgenics, but the protection from lepidopteran resulted in the gain of fecundity in transgenics. They suggested that the wild sunflowers and weedy populations near to the cultivated transgenic sunflowers would render recurring events of "gene flow" from the transgenics and it could have harmful effects on the native lepidopteran herbivores and other populations of coleopteran and dipteran herbivores (Snow *et al.*, 2003). One of the most generalized sunflower constraints caused by Arthropoda is stand establishment failure due to soil larvae: mainly of Coleoptera, Elateridae, and Lepidoptera. These herbivores which feed on seedling stems and roots at different levels all correspond to polyphagous species. Insects that eat the aerial parts of plants, including some aphids and white flies, can be particularly important during early stages of crop development. A small number of these predators are exclusive to sunflower and are only found at the center of origin (Charlet *et al.*, 2009; Lopez, 2002).

The relative importance of crop plagues constitutes a dynamic situation that technological developments can do much to change. This does not only relate to improved control methods but also to general changes in the ecosystem. With the increase in no-till surfaces, two previously unnoticed snails of genus *Deroceras* have recently become limiting factors for Sunflower crops (Carmona, 2001). On the other hand, CpT1 could improve stand establishment in cases in which failure is due to Coleoptera of genera *Agriotes sp.*, *Melolontha sp.*, *Anoxia sp.*, and Orthoptera of *Calolampra spp.* and *Teleogryllus spp.* Larvae of these species exhibit subterranean habits and eat plant roots at different stages of crop development, causing the death of seedlings in early attacks (Charlet *et al.*, 2009; Lopez Bellido, 2002). To achieve the required impact at crop establishment, the expression of CpT1 toxin should take place early in crop development and involve concentrations that are lethal for the plague. Seedlings are very sensitive to the loss of certain of their parts, so it is therefore important to stop damage as early as possible at the beginning of the attack. Two beetles cause economically important damage in North America (Charlet and Brewer, 2009; Soyars *et al.*, 2018).

According to Cantamutto & Poverene (2007 & 2010) genetic engineering include GM crops that express gene fragments from insecticide proteins of *Bacillus thuringiensis* (*Bt* endotoxins called Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca, Cry1Fa, cry3Aa, and others), the *Vigna unguiculata* trypsin inhibitor (CpT1), lectins, and other metabolic inhibitors. The most widespread *Bt* proteins show strong activity against Lepidoptera, although some bacterial variants have also proven effective also against Diptera (*B. thuringiensis var. israeliensis*) and Coleoptera (*B. thuringiensis var. tenebrionis*) (Cantamutto & Poverene, 2007). There is strong specificity in the action and expression *Bt* endotoxin. Not all the genes that codify *Bt* proteins are expressed in the different plant species (Cantamutto & Poverene, 2010). Similarly, nor all the Lepidoptera found in a crop are controlled by the same event. On the other hand, the CpT1 agent is very active against Coleoptera and Orthoptera (Boulter *et al.*, 1989) and is already available in GM crops. Modern biotechnological strategies incorporate the expression of a carrier to improve the toxin penetration and its influx into the insect's *haemolymph* (Fitches *et al.*, 2004, Soyars *et al.*, 2018).

Biotechnology could improve this control by helping to develop insect-resistant GM sunflower. However, sustainable management calls for a complete knowledge of the biology of the target pest and its relationship with other components of the agro-ecosystem. A polyphagous insect *Helicoverpa armigera* is reported to cause 20-25% yield losses in sunflowers and sometimes up to 40-70% in severe conditions (Rana and Mahalik, 2008). Westdal (1975) found that the sunflower beetle *Zygogramma exclamation* is reduced seed production in sunflower as much as 30%. Lectin or proteinase inhibitor genes have been used to engineer sunflower with insect resistance (Schuler *et al.*, 1998; Burke and Rieseberg, 2003; Mukhtar, 2009; ÇALIŞKAN, 2016). *CryIF*-transgenic sunflowers were obtained with a *CryIF* gene ("Bt" gene) isolated from *Bacillus thuringiensis* which conferred resistance against *Spilosoma virginica* and *Rachiplusianu*. Compared to the control, increased tolerance of transgenic plants against larvae at the seedling and pre-flowering stages were found during the feeding assay with transgenic leaf discs. *CryIAC* gene was used to develop a transgenic line of *Bt* sunflowers by Pioneer Hi-Bred and Dow AgroSciences which produce *CryIAC* protein that is lethal to *Lepidopteran* (moth) larvae (Snow *et al.*, 2003). According to Snow *et al.* (2003) report the transgenic plant showed considerably more inflorescences with more mature seeds in more inflorescences and higher number of viable seeds per plant as compared to non-transgenic controls.

The ideal GM technology should be environmentally friendly, with a wide spectrum of activity with respect to the target insects, but with few if any effects on beneficial insects (Hilder and Boulter, 1999). The GM sunflowers released into the

environment and authorized for research include two groups of events for insect control. The reported Lepidoptera-resistant varieties express the *Bt* insecticide protein, which is codified by the *CryIF* gene (Cantamutto & Poverene, 2007). If expressed in the early stages of crop development, this could be a valuable tool for controlling polyphagous moth larvae of genera *Agrotis* and *Euoxa*, which are present in the main sunflower growing regions (Charlet *et al.*, 2009). For *Suleima helianthana*, which bores sunflower roots and stems in North America, control through the use of GM varieties is difficult to justify because the damage caused is seldom significant (Charlet and Brewer, 2001, Soyars *et al.*, 2018). *Bt* proteins could also offer excellent possibilities for controlling insect damage to aerial tissues. Lepidoptera that causes important crop damage include *Heliothis spp.*, *Helicoverpa spp.*, *Diabrotica spp.*, *Spilosoma spp.*, *Colias lesbia*, *Rachiplusianu* and *Vanessa cardui*. These species could be controlled through GM technology based on *Cry1* variants of the *Bt* gene (Cantamutto & Poverene, 2007 & 2010). As these species are highly polyphagous, refuges to prevent the selection pressure for insect resistance would not be indispensable, except in cases where all the crops in a given region were GM varieties with the same expression of *Bt* proteins (Cantamutto & Poverene, 2007 & 2010; Soyars *et al.*, 2018).

Herbicide Tolerance Development Effort

The success of selection greatly depends on the presence of genetic variability for the traits concerned. Considering the relatively narrow genetic base of cultivated sunflower, the existing genetic resources are an invaluable source of variability, which can be used for the introduction of agronomically important genes to improve the quality and economic value of sunflower crops. Considering, broomrape, wild *Helianthus* species are a most important source of resistance, but other sources, such as open pollinated varieties, and different gene pools of cultivated sunflower developed in research institutions around the world, have also been used for the introduction of broomrape resistance. The first sunflower varieties resistant to broomrape were developed by the Soviet breeders in the first half of the 20th century (Gorbachenko, 2011). Those were local varieties, such as Saratovsky 169, Zelenka and Fuksinka, resistant to race A. Especially important are varieties resistant to broomrape, like Zhdanovsky 6432, Zhdanovsky 8281, and Stepnyak, created by academician Zhdanov and developed at the Saratov experimental station when the occurrence of broomrape race B threatened to jeopardize sunflower production (Gorbachenko, 2011). According to Gorbachenko (2011) one of the largest collections of open-pollinated varieties is maintained at the Vavilov All-Russian Institute of Plant Genetic Resources from Saint Petersburg, Russia containing 400 genotypes.

The hemi-parasitic weed broomrape (*Orobancha spp.*) which constitutes an important crop limitation in the Mediterranean region could be effectively controlled in sunflower if herbicide resistant varieties were available (Nandula, 1998). This strategy has proven useful in others crops and could be improved if the herbicide were brought with the seed, because broomrape affects the roots before emergence. At present, control strategies tend to use a specific gene mechanism which is also obtained in wild species (Fernández-Martínez *et al.*, 2000; Labrousse, 2002). However, the continuous appearance of new races of the weed means that a process of constant renewal of resistance sources is required to maintain these control strategies. Some herbicides that are members of the imidazolinone and sulfonyleurea families including imazethapyr (Gressel *et al.*, 2013) inhibit AHAS (Group B) and are therefore useful for controlling *O. aegyptiaca* and *O. cernua* (Nandula, 1998; Alonso *et al.*, 1998). Some other groups have also proven effective against this weed, including glufosinate-ammonium (Valkov *et al.*, 1998) and glyphosate (Collin, 1999). This may also be possible with GM sunflower because tolerance to these herbicides is currently under investigation (Kaya, 2015; kaya, 2021; Soyars *et al.*, 2018).

Although still not widely disseminated, the GM technology that has been developed for sunflower includes tolerance to glyphosate and glufosinate-ammonium herbicides. Both of these herbicides are systemic and neither has residual effects upon the soil. Glyphosate is used on a very large scale and is relatively inexpensive, but reiterated use can promote weed resistance (<http://www.weedscience.org/summary/MOASummary.asp>). The need for RR sunflower to facilitate crop management in no-till systems seemed to disappear with the discovery of genes capable of conferring resistance to herbicides that belong to the imidazolinone (IMI) and sulfonyleurea groups and which were found in wild sunflower populations in Kansas, under field conditions to inhibit the hydroxyacetic acid synthetase (AHAS) enzyme (Baumgartner *et al.*, 1999; Kolkman *et al.*, 2004; Soyars *et al.*, 2018; White *et al.*, 2002). By transferring these mutations to crop Germplasm in the USA and Argentina, seed companies created non-GM sunflowers, under the commercial name of Clear field, that were tolerant to both imazapyr and imazamox (Zollinger, 2004). Tolerance gene expression in these new

varieties allows herbicide application at advanced stages of crop development, thus controlling the majority of weeds (Kaya, 2015; Yalcin, 2017).

There are many cases in the world of weed populations displaying resistance to herbicides that inhibit AHAS, 95 cases in 63 genera, including *Helianthus*, and also to other herbicides; this points to the need to keep on searching for new control strategies (Presotto, 2012). Weeds resistance to the chemical group of herbicides which could be used in sunflower, under different management strategies, including two GM varieties at present under research. Given the absence of glufosinate-ammonium resistance among weeds, a good long-term strategy could involve incorporating this tolerance through GM sunflower. Moreover, two homologous “bar” and “pat” genes that codify the phosphinothricin acetyltransferase (PAT) enzyme have been shown to be safe for this purpose as they do not cause allergy and are rapidly degraded in the intestine (H erouet *et al.*, 2005; Yalcin, 2017; Soyars *et al.*, 2018).

Recent sunflower genome assembly will definitively help identify the possible candidate genes involved in resistance to broomrape as well as their function. So far, only a few authors have exploited the sunflower genome sequence in their molecular research study, as is the case with the exploitation of various -omics techniques in examining sunflower-broomrape interaction. The progress in -omics techniques and powerful statistical tools in big data analysis should be exploited to the fullest for conducting more advanced and detailed research in revealing mechanisms underlying complex interaction between sunflower and broomrape as well as characterizing resistance pathways in sunflower. Unfortunately, there have been no reports on the examination of epigenetic mechanisms in sunflower resistance. As a new field of research, it would be very useful to examine to what extent epigenetic mechanisms influence resistance in sunflower, considering that the DNA methylation status plays a crucial role in regulating *Phelipanche ramosa* seed germination during conditioning period, by controlling the *strigolactone*-dependent expression of PrCYP707A1 (Lechat *et al.*, 2015). Moreover, sunflower broomrape genome was recently sequenced with the addition of 20 transcriptomic experiments for the annotation of the genome sequence (Badouin *et al.*, 2017). This can be a starting point for identification of avirulence genes in broomrape as interactors with virulence genes in sunflower, a better insight into resistance mechanisms developed by sunflower as well as unravelling new resistance genes.

Developments in gene editing techniques could lead to the quicker design of superior broomrape resistant genotypes. Powerful CRISPR-Cas9 technique was successfully used for mutagenesis of the CCD8 (Carotenoid Cleavage Dioxygenase 8) gene, *strigolactone*-biosynthesis gene, in order to create *Phelipanche aegyptica* resistant tomato lines (Bari *et al.*, 2021). Another gene silencing technique, virus-induced gene silencing (VIGS) was used to induce trans-silencing of PaCCD7 and PaCCD8 genes in *P. aegyptica* for significant reduction in the number of parasite tubercles attached to *Nicotiana benthamiana* roots (Aly *et al.*, 2014). New gene editing techniques can be difficult to apply in sunflower breeding, mainly due to the difficulties that occur during plant regeneration and low numbers of obtained transgenic regenerants per assay. Thus, the first step for use of the modern gene editing techniques would require the establishment of improved basis for transformation, which could be beneficial in the development of durable broomrape resistance in sunflower.

3. DEVELOPMENT OF ABIOTIC STRESS TOLERANT TRANSGENIC SUNFLOWER

Heavy Metal Tolerant Development Effort

Yeast metallothionein gene (*CUPI*) from yeast was incorporated into sunflower to evaluate tolerance of transgenic plants to heavy metals at the callus stage and selected heavy metal tolerant lines of the transgenic sunflower calli. The results showed use of transgenics to obtain abiotic stress tolerance in sunflowers (Watanabe *et al.*, 2005; Soyars *et al.*, 2018). LBA4404 strain harboring T-DNA containing dsRNA-suppressor of proline dehydrogenase gene, produced based on the *ProDHI* gene of Arabidopsis, was integrated into the genome of sunflower plants transformed *in vitro* and *in planta* to increase sunflower tolerance level to water deficiency and salinity (Tishchenko *et al.*, 2014; Soyars *et al.*, 2018).

Drought Tolerant Development Effort

Sunflower is categorized as a low to medium drought sensitive crop. It has been found that both quantity and distribution of water has a significant impact on achene and oil yield in sunflower (Fereris *et al.*, 1986; Andrich *et al.*, 1996; Krizmanic *et al.*, 2003; Reddy *et al.*, 2003; Iqbal *et al.*, 2005). However, oil quality of sunflower has not been significantly affected by drought treatment (Petcu *et al.*, 2001). During 1997-2017 years, crops of sunflowers have extended in EU

Mediterranean area due to its capacity to adapt to dry environments. Although sunflower was moderately tolerant of drought, production is strongly influenced by the presence of water stress, which is found fairly regularly. In fact, drought is a permanent feature in many developing areas and may also periodically influence the economy of the Europe and USA. The late stress is typical of Mediterranean environments and the crop during the flowering-grain filling period is affected by drought during the development of the dry season. The threats from the trend in the climate render this property ever more important. Unfortunately, useful knowledge concerning the improvement of the species to environments with trophic, especially water; limitations are still scarce and fragmentary (Panković *et al.*, 1998, 1999, 2000, 2001; Soyars *et al.*, 2018).

Genetic improvement for drought tolerance has been limited, by the lack of simple screening technologies to assess the drought tolerance of genotypes, obliging the breeders to utilize methods involving selection for yield and its stability, with repetitions over many locations and years, which are expensive, laborious and time consuming (Blum, 1989). In order to analyze germplasm in relation also with agronomic and physiological observations on population offspring in field or controlled conditions, several molecular markers could be used either for SNP or for general mapping (Streit and SA, 2012). SNP will help to detect polymorphism hidden until now but potentially important in *Helianthus* bio-diversity and candidate genes functional properties. This will result in a candidate gene approach combined with QTLs mapping for the exploitation of desirable traits issued from yet under-exploited germplasms either from cultivated (Panković *et al.*, 2000; Hervé *et al.*, 2001) than wild *Helianthus species* (*Helianthus argophyllus*) crossed with sunflower. Introgression of traits conferring higher drought tolerance would therefore lead to an improvement of this crop into a more economically optimized and ecologically well-adapted system for the production of renewable resources.

Development of Mineral nutrition Sunflower Lines

Sunflower is a highly nitrogen-dependent crop which, unlike soybean, does not perform nitrogen fixation (Ali, A., & Ullah, S. 2012). This limits its growth and development in poor soils and under no-till situations, where it is necessary to add nitrogen fertilizers (Díaz-Zorita & Fernández-Canigia, 2009). GM sunflower has been put forward as a possible way to improve nitrogen absorption. In plants, ammonium absorption, which is an alternative pathway to the nitrogen cycle, is performed through the *glutamine synthetase* (GS) enzyme. However, in darkness and with a low available C:N ratio, some variants of *asparagine synthetase* (AS) enzyme, coded by HAS1 and HAS1.1 genes provisionally store N as asparagine, thereby preventing ammonium intoxication (Cantamutto and Poverene, 2007). In GM plants, AS can substitute GS under conditions that limit its activity; such as in *Medicago truncatula* (Carvalho *et al.*, 2003) and act as an alternative N-storing metabolic pathway (Ferrario Méry *et al.*, 2002). AS expression in GM sunflower might therefore improve N metabolism and contribute to a more efficient use of this element.

Development of High Oil Quality Sunflower Lines

Traditional sunflower oil has been the major polyunsaturated oil used for many years in human nutrition to replace saturated fat, in an attempt to reduce cardiovascular diseases (Carmena *et al.*, 1996). The quality of sunflower oil is generally associated to the relative content in linoleic fatty acid. However, it has been demonstrated that diets including high consumption of monounsaturated oils are as effective as those rich in polyunsaturated oil in lowering cholesterol (LDL-C), but in contrast to the effect of polyunsaturated diets, the monounsaturated diets do not lower HDL-C (Delplanque, 2000). Thus, the development of new selected sunflower seeds producing enriched in oleic acid (Lacombe and Bervillé, 2000) at the expense of linoleic acid has made sunflower oils highly competitive compared with other traditional predominant monounsaturated oils (Delplanque, 2000). Moreover, the present trend in human diet is to decrease the consumption of the saturated palmitic and stearic fatty acid. To identify sunflower germplasm with reduced saturated fatty acid composition, a total of 884 cultivated sunflower accessions has been screened (Vick *et al.*, 2014). The genetic analysis of selected plants with low saturated fatty acid content indicates that the trait is dominant (Vick *et al.*, 2014). In addition, preliminary data suggested that the content of these fatty acids can be reduced introducing genes from wild perennial progenitors (e.g., *H. giganteus*) into cultivated sunflower (Seiler, 2011).

The increase of alternative fatty acid contents in the oil has stimulated new markets, thus providing new interest in growing sunflower (Gielen, 1992). Mutants affecting seed oil fatty acid composition are of great value leading to novel oil composition (Lacombe and Bervillé, 2000). In sunflower several different mutants have been isolated using chemical and physical mutagens (Soldatov, 1976; Ivanov and Ivanov, 1985; Garcés *et al.*, 1992; Osorio *et al.*, 1995; Miller and Vick,

1999; Pérez-Vich *et al.*, 1999 & 2000), however, little is known about the molecular nature of these mutations. Nevertheless, candidate genes from the fatty acids biosynthetic pathway have been recently mapped using molecular markers (Hongtrakul *et al.*, 1998; Lacombe *et al.*, 2000; Pérez-Vich *et al.*, 2000 and Lacombe and Bervillé, 2001) that can serve as starting point for chromosome walking or chromosome landing to clone the corresponding gene.

Modifying oil quality is crucial as it is one of the edible oils worldwide known for its salubrious quality and lipid peroxidation (Moschen *et al.*, 2014). Many sunflower lines have been developed with elevated saturated fatty acid content with greater than 25% of fatty acids compared to 12% in normal sunflower using physical or chemical mutagenesis. Osorio *et al.* (1995) developed CAS-3 and CAS-5 mutants with high amount of stearic acid and palmitic acid contents respectively. Fernández-Martínez *et al.* (1997) reported CAS-12 mutants with high palmitic acid and oleic acid contents. Fernández-Moya *et al.* (2002) developed CAS-14 mutants with upto 37% stearic acid content. Velasco *et al.* (2008) used ethylmethane sulfonate as a chemical mutagen and obtained M2 seeds from a single M1 plant with 5-39% palmitic acid content. 10-30% of palmitic acid was obtained from the progenies of all selected M2 seeds.

4. BRIEF HISTORY OF SUNFLOWER RESEARCH IN ETHIOPIA

In Ethiopia, sunflower improvement started in the late 1960's and during that time three varieties namely Russian black, Hesa and Pop-158 were recommended for production at national level. Structured research work on sunflower has been conducted since 1980s at Hawasa Agricultural Research Center, which was a coordinating center till 2004 to release open-pollinated varieties with desirable agronomic traits. In Ethiopia, according to Hiruy (1985) progress report on weed-sunflower critical period study for three consecutive years (1983-1985) shows that the critical time of competition between weed and sunflower starts during early crop establishment period and extends up to early budding stages of the crop. Chemical screening trials also conducted in order to select best herbicides. The result revealed that Pendimethalin at 3.0kg a.i/ha followed by twice hand weeding was the best recommended treatments without phytotoxicity on sunflower. Sunflower variety screening trial for broomrape tolerance also conducted for two years (1984 and 1985) at shambu area; out of 17 varieties exposed to natural infestation no orobanche species were recorded from Russian variety WIR-3001 (Hiruy, 1985). According to Hiruy (1985) there were no herbicides screening for tolerance trial conducted after this report in Ethiopia.

During 1990's, the state farms was lost interest in producing sunflower due to different reasons; mainly the severity of bird damage in the production areas, disease occurrence such as downy mildew and sclerotia and lack of strong research program and follow up (Hiruy, 1985; Tesfaye *et al.*, 2016). Due to the above challenges the then IAR-management decided to cease the research program altogether and all germplasm materials had been kept in safe at the then PGRC/E (now Ethiopian Biodiversity Institute EBI). A few years after there was growing interest from private sector to produce sunflower and thus Ethiopian Institute of Agricultural Research (EIAR) has started to re-initiate sunflower research by assigning Holeta Agricultural Research Center (HARC) to coordinate the program (Getinet Alemaw and Nigussie Alemayehu, 1992; Tesfaye *et al.*, 2016; Tesfaye & Mengistu, 2021).

EIAR, National mid and high land oil seeds research program started by acquiring and evaluation of germplasms from Ethiopian Biodiversity Institute (EBI). In 2005, the first open-pollinated variety namely Oissa was released from Hawasa agricultural research center and in 2014 Ayehu was released from Adet Agricultural research center. Since 2010, a number of hybrid sunflower varieties have been registered through conducting adaptation trial at various locations in collaboration with different private companies. According to the reports from variety evaluation committee, the registered hybrid varieties were found to be adaptable to wide Agro-ecology and gave better productivity and their oil content ranged from 17-31 Q/ha and oil content 37-51 %, respectively (Ayana *et al.*, 2016). Different breeding works was done including germplasm characterization, along with standard checks (*Oissa and Russian black*) and pipeline sunflower genotypes for common quantitative phenotypic characters.

Recent study done by Sakatu *et al.* (2020) on sunflower agronomy shows that all parameters tasted were significantly different for varieties tasted. The main effect of fertilizer rate significantly affects all yield and it contributing traits except oil content. Finally, the study identified out of five tasted fertilizer application rates, 34.5 kg N/ha and 34.5 kg P₂O₅ /ha becomes profitable and recommended for sunflower production at the study area. Besides, the author's recommended that this experiment would be further confirmed in other areas and soil types for sunflower production in general. Accordingly, astonishing result will be expected from the undergoing holistic research efforts from the sunflower

breeding program at national level soon in order to satisfy the existing gap. Now a days genetic variability study was done by a few MSc students with promising results and this may be starting point for further breeding strategy as sources of gene for desired characters to improve sunflower.

Current Situation and Challenges Globally

A progress is still being made in efficiently transforming sunflower crops but stable transformation of sunflower plants is just yet time-consuming in generating homozygous lines and in regeneration of tissue. Also, sunflower has a long-life cycle and transient expression of genes can be an alternative method in elucidating molecular mechanisms such as function of promoters, regulation of gene, subcellular localization of proteins, protein stability, protein-protein interactions and small RNA function (Manavella and Chan, 2009). Despite of this constraint, several studies by developing transgenic sunflowers are still being conducted. Wild sunflower species provide greater contribution as a rich source of genes in crop improvement to bring about economic viability in cultivated species as major oilseed global crop (Seiler and Fredrick, 2011).

Having a narrow background in domesticated sunflowers with deficient genes, discovery of unique genes from wild sunflower plants is indispensable and is still underway. This could help in developing transgenic sunflowers with desired traits from wild population. Clear field and Express Sun technologies saw restriction on growing of genetically modified crops for “not being biotech product”. Genetically modified crops have always been a matter of debate and public acceptance regarding this remains divided with some people being reluctant on the use of biotechnology in crop amelioration. In Ethiopian, a few sunflower accessions we have with different undesirable traits to be a cultivated sunflower; this may lead narrow genetic background in domesticated sunflowers with deficient genes. A few genetic variability studies confirmed that Ethiopian sunflower germplasm falls three to five cluster and this limits the starting energy for any breeding works.

Future Prospects of Sunflower Research in Ethiopia

Sunflower production and area coverage is very small in Ethiopia. However, the growing demand for the seed is increasing by private farmers as well as some small-scale farmers due to the demand of mega oil mill factories. Nowadays, consumers especially higher and middle classes prefer to use introduced sunflower oil despite its price is relatively high compared to palm oil. This depicts that production of sunflower on large scale level will be profitable. In other words, we can save foreign currency through import substitution. In addition to oil, there is an eminence potential to grow confectionery sunflower seed for feed and utilizing its stem for fuel; and also, now a days sunflower flower has high demand in international market including 2020 Olympic ceremony used ornamental type of sunflower. So, our country can benefit from floriculture industry by exporting ornamental type of sunflower to the world market. Despite its diverse relevance of the crop, the available improved sunflower varieties are very limited and thus the improvement of sunflower should proceed at the same pace with its demand.

Enhancement of the existing germplasms through recurrent selection, hybridization and introductions is necessary for the improvement effort of the crop. The current effort of developing high yielding open-pollinated varieties should be strengthen including other quality parameters such as improvement of oil content and oleic acid supported by modern biotechnological tools. Collection and characterization of accessions from untouched areas of Ethiopian should not be undermined since we have relatively less population of sunflower in a gene pool. Transgenic technology holds imperative role in sunflower breeding and exerts strong promises to increase yield, oil content, pest resistance, stress tolerance and production of biopharmaceutical proteins. Although having improved transformed techniques in sunflower, more efficient transformation protocol needs to be started for generating increased success rates in obtaining transgenic sunflowers as well as search for noble genes with elite traits in developing transgenic crops does remain outward. Traits that are being studied in sunflower for environment release is sparse. Sunflower is known to have a high exposure to gene flow ultimately generating continuous variability. Strict environmental monitoring is inevitable to preclude undesired outcomes. In the near future the national oilseeds research needs a holistic planned to develop hybrid varieties (forthcoming focus) in addition to OPV and the use of modern tools of plant breeding with the hope that all necessary conditions such as trained man power and facilities provided or availed for the research.

5. CONCLUSION

Biotechnology can speed up plant breeding scheme, with many of the techniques complementing rather than substituting conventional methods and some biotechnological products have had a strong impact upon production systems because they have also facilitated crop management. Globally, the role of genetic engineering in improvement sunflower have done a big effort to combat biotic and abiotic stresses. Molecular biology research with a focus on transgenic sunflowers to develop pest resistance, herbicide resistance, increasing oil yield of sunflower holds indispensable as well as its study on ecological impact is contradicting. In an era marked by political and societal pressure to reduce the use of pesticides, crop protection by genetic improvement provides a promising alternative with no obvious impact on human health or the environment. Genome editing is one of the genetic levers that can be adopted, and disease resistance is frequently cited as the most promising application of CRISPR/Cas9 technology in agriculture. CRISPR technology now a day giving a solution for different production constraints of different crops including sunflower as discussed above. Sunflower GMO have been developed with increased oil quality and quantity as well.

Sunflower is one of the major sources of oil in the world and widely spread and grown oilseed crop of the world. In Ethiopia, sunflower improvement started in the late 1960's and now a days Oissa and Ayeahu are two OPV varieties registered for production. Although sunflower is not widely grown in Ethiopia, the country has immense potential for sunflower production in different region. The research system has been working on development of variety by different strategic ways like germplasm acquisition and characterization, registration of hybrid varieties, OPV development effort and also crop management recommendations and quality analysis are not forgettable efforts in the system. Generally, Sunflower research in Ethiopia has to plan to develop sunflower varieties using recent modern tools of biotechnology especially CRISPR/Cas9 in the future in order to combat dynamic environmental condition and to fasten the existing sunflower breeding for the betterment of future generation as well.

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