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Biomedical Applications and Secondary Metabolite Profiling of *Hyoscyamus niger* and *Sesamum indicum* Seed, Root and Hairy Root Cultures

Dissertation

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
ATCC	American Type Culture Collection
APS	Ammonium Persulfate
BH	Black Henbane
Bb	Bubble bioreactor
CDW	Condence Decoction Waters
C. elegans	*Caenorhabditis elegans*
CENIAP	Centro Nacional de Investigaciones Agropecuarias
cm	Centimeter
^{o}C	Degree centigrade
CP	Chloroplast
DMSO	Dimethyl sulfoxide
DO	Dissolved oxygen
DW	Dry weight
df	Degree of freedom
E. Coli	*Escherichia coli*
Eos	Essential oils
EtBr	Ethidium Bromide
ELSD	Evaporative Light Scattering Detector
EST	Expressed sequence tag
FAD	Fatty Acid Desaturase
FW	Fresh weight
g	gram
GA3	Gibberellic acid
GBS	Genotyping by Sequencing
GI	Growth index
HPLC	High-performance liquid chromatography
HPLC-MS	High-performance liquid chromatography- Mass Spectrophotometer
HR	Hairy root
HRCs	Hairy Root Cultures
ISSR	Inter Simple Sequence Repeats
KRI	Kurdistan Region of Iraq
LB	Lysogeny broth
LC-MS	Liquid chromatography–mass spectrometry

LC	Lethal concentration
LT	Lethal Temperature
MAS	Marker-assisted selection
mg/L	Milligram per litre
Min	Minute
mm	Millimeter
mL	Milliliter
µg	Microgram
µL	Microliter
µM	Micro molar
MS	Murashige and Skoog
NGM	Nematode growth media
NMR	Nuclear magnetic resonance
PD	Petri dish
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
RAPD	Random Amplified Polymorphic DNA
rpm	Rotation per minute
SAD	Stearoyl Acyl Desaturase
SSR	Simple sequence repeats
T-DNA	Transferable-DNA
TF	Transformation Frequency
UV-Lamp	Ultraviolet lamp
ZJK	Zana Jamal Kareem
%	Percentage

SUMMARY

Nature has been known to be a rich source of pharmacologically essential compounds over the years. Phytochemical studies have revealed a large number of natural products with a unique chemical structure and important medical potentials. *Hyoscyamus niger* L. is considered as one of the important medicinal plants during the history and *Sesamum indicum,* which has been known for a long time as an oil crop Lately, several studies proved their biomedical activity. This study aimed to prove new pharmacological uses of these two medicinal plants and to develop *in vitro* protocols for improving the yield and quality of the secondary metabolite and accurate system for examining their biomedical activity.

Germination efficiency and dormancy in black henbane and *Sesame* were tested in the laboratory for 7 varieties of *H. niger* and 25 varieties of *S. indicum* using different gibberellic acid concentrations in mg/L. The results showed that GA3 is one of the most important factors that affect breaking seed dormancy. Seed germination was more pronounced in the dark, and there was an increase in seed germination percentage with increasing GA3 doses up to 250 mg/L, which gave the highest germination percentage. The hairy root culture accumulates phytochemicals is most similar in quantity than the intact plants with stable genotype and biosynthesis. Hairy root cultures of 7 varieties of *H. niger* and 25 varieties of *S. indicum* were generated using *Agrobacterium rhizogenes* ATCCA15835 and A4 strains. Two different sterilization protocols were used to examine the viability of the explants and the response capability; aseptic protocol and non-aseptic protocol. For the aseptic protocol, 100% transformation frequency was recorded, while the highest response for the non-aseptic protocol was 38%.

The transformation frequency (TF) of both *A. rhizogenes* strains was examined for all varieties of both plant species. The highest TF using *A. rhizogenes* ATCCA15835 for both plants were 100%, and the lowest TF was 88% and 31% for *H. niger* and *S. indicum* respectively while for *A. rhizogenes* A4 strain the highest TFs were 57% and 68% for *H. niger* and *S. indicum* respectively, and the lowest TF was 13% for *S. indicum*. The hairy root suspension culture was established for all plant varieties, the highest hairy root biomass yield for *H. niger* using *A. rhizogenes* ATCC15835 and A4 strain was 10 g/L and 7g/L respectively, while biomass yield for *S. indicium* using *A. rhizogenes* ATCC15835 and A4 strain was 9 g and 7 g respectively. Bubble column bioreactor has been designed and used for hairy root initiation. The highest dry weight biomass obtained from bioreactor propagation was 14 g/L for *H. niger* and 12 g/L for *S. indicum* which was significantly different compared with HRCs grown in a shaking flask culture with fixed air and agitation which was 9 g/L for *H. niger* and 8 g/L for *S. indicium*.

Different protocols have been used to examine the biomedical activity of *H. niger,* and *S. indicum* varieties, one of the most accurate method is using *Caenorhabditis elegans* nematodes as an advanced model. The methanolic extract of the plant varieties was prepared from lyophilized seed, root, and hairy root culture. Moreover, essential oil and thermal release were examined. The highest mortality from *H. niger* essential oil was 99% at 500 µg/mL, and the LC_{50} ranged from 33.6 to 58.1 µg/mL for the varieties KRI Pinjwen, and Iran Isfahan respectively.

Hairy root methanol extract for both *H. niger* and *S. indicum* shows significant response against *C. elegans*. Nine concentrations of 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500 µg/mL were used to conduct this study. The lethal dose LC_{50} for *H. niger* ranged between 39.7, and 48.2 µg/mL, and for *S. indicum* ranged between 299.4, and 367.1 µg/mL. While for *H. niger* root extract ranged between 136.6, and 319.5 µg/mL but for *S. indicum* was considered as undefined. Regarding both plant species seeds, two types of the extract were prepared, crude extract and fexed oil extract. The crude extract from *H. niger* showed a significant response and LC_{50} ranged between 41.0 and 92.2 µg/mL, while for *S. indicum* was considered as undefined, moreover, the fixed oil showed significant mortality at LC_{50} for *H. niger* ranged between 57.0, and 66.9 µg/mL, and for *S. indicum* 427.7 µg/mL for Indian 7 varieties.

The thermal release was examined as a protocol for the antinematode activity of *H. niger* and *S. indicum* seeds against *C. elegans* nematodes. Several heating degrees were examined for one hour at 0, 50, 60, 70°C, 80, 90, and 100°C. The results were surprising, in which highly significant mortality 100% was recorded for all varieties at 100^{o}C, and 94% at 90^{o}C and the LT_{50} ranged from 77.4, 77.3, 72.9, 80, 82.6, 79.8, and 78.11°C for the varieties KRI Hawraman, KRI Hasanawa, KRI Pinjwen, KRI Daray Mar, Iran Takhte, Iran Isfahan, and Germany 1 respectively, while *S. indicum* showed no a significant response.

Secondary metabolite profiling of *H. niger* and *S. indicum* varieties were studied using HPLC-ELSD chromatograms, for chemical analysis of hairy root exudates from both bioreactors and flask culture. The functionality of the bioreactor was demonstrated by the growing hairy roots of both plants; then, the exudates were collected from hairy roots grown in bioreactors and shaking flask cultures then analyzed by HPLC-ELSD. Differences among metabolic profiles for different varieties of the same species revealed substantial chemical diversity, verifying the need to examine multiple plant varieties while optimizing metabolite production in hairy root cultures. Different classes of lignans as sesamin, sesamolin, sesaminol, pinoresinol, and sesamol were detected in *S. indicum* using HPLC-MS. The results show that all three different plant tissues for all varieties contain lignans. Furthermore, the production of the hairy root Sesamin was higher than the normal root.

The basic level of biodiversity is genetic diversity, which means the variability of genotypes in plant species. The genetic diversity of *H. niger* and *S. indicum* was examined using DNA sequencing; for this reason, four specific primers, four genes were amplified from *H. niger* and *S. indicum*. The genes were *HNTR 1, HNTR 2,* and *FAD2, SAD,* respectively. The multiple sequence alignment results showed that most variations were detected among the samples of *H. niger* when compared to the reference strain. However, the amplified genes in the *S. indicum* in different isolates showed fewer variations.

CHAPTER ONE

1. INTRODUCTION

The medicinal plants have been known for their antimicrobial activity since ancient times (Thakare, 2004). Medicinal plants belonging to the cultural heritage of indigenous people. Humanity has shown the importance of medicinal plants. 80% of individuals globally are depended primarily on traditional health care, in particular, herbal medicines (Shanley and Luz, 2003).

Medicinal properties of plants were mostly discovered in many cultures through trial and error, but mostly it has been used by peoples who believe in it and often intertwined with religion and mythology. Medicinal plants make significant contributions to the improvement of prominent medicinal drugs that currently have been used (Fuku, 2018). In the control or avoidance of illnesses like diabetes, heart problems, and different cancers, the application of herbal medicines have always been of great importance (Mohanta *et al.*, 2003).

Traditional medicine means understanding, abilities, and practices based on indigenous theories, values, and experiences of different cultures used to sustain and prevent and diagnose human disease (WHO, 2002-2005). Anthelmintic medications are used to control helminth parasite infections. The demand for the latest and efficient anthelmintic is huge, given that the chemical drugs used in helminth control are costly, and most of them lose their effect in 20 years owing to the issue of resistance (Sant'anna *et al.*, 2013).

Hyoscyamus niger is widely spread in Europe and Asia and is commonly known as henbanes. The plant has anti-spasmodic, sedative, and analgesic characteristics (Sajeli *et al.*, 2006). BH has pharmacological effects, such as a bronchodilator, antisecretory, relaxing urinary blood, spasmolytic and hypnotic properties, dilatory properties of pupils, sedative properties, and antidiarrheal properties, as well (Alizadeh *et al.*, 2014). This foul-smelling shrub is the source of the narcotic alkaloids hyoscyamine, scopolamine, and atropine. Its name comes from the Anglo-Saxon Henn (chicken) and Bana (killer) because they become paralyzed when fowls consume the seeds of this crop (Schultes and Smith, 1976; Carter, 2003, Dulger and Dulger, 2015). It is determined in past studies, and field trips that the aqueous components produced from the *H. niger* plants were applied for spilling over the larvae from the human eye, so locally the plant called "shed-helmet" (Dulger and Dulger, 2015). Black henbane Black henbane has been used as a medication for all traditional drugs for the last decades. It is a herbal medication but may inadvertently or deliberately cause intoxication. Some alkaloids such as Hyoscyamine,

Atropine, Tropane, and Scopolamine are present in all parts of Black henbane (Alizadeh *et al.*, 2014).

Sesame, *Sesamum Indicum* L. is the oldest oil-seed crop and has been cultivated in Asia for more than 5000 years (Bisht *et al.,* 1998). Sesame oil has medicinal uses and is used for the manufacture of liniments and ointments in British pharmacopeia. In Africa and Asia for a multitude of diseases, various sections of sesame plants are included in indigenous drugs (Bedigian, 2010). Sesame seeds includes the essential fatty acids, for instance, linoleic acid, olic acid, palmitic acid, during the lignan glycosides such as sesamine, sesaminol, sesamol, sesamolinol, and sesamolin lignan concentrations. Several studies have shown that sesam has a health-promoting property. Seeds and oil are known as traditional health products and have been used for a long time in ancient Chinese medicine (Kanu *et al.*, 2010).

Lignans are structurally and functionally varied phytochemicals that can be biosynthesized by various plant species. They got great attention in leading compounds in new tumor therapy drugs, thus reducing the risk of life-style non-communicable diseases (Satake *et al.*, 2015). Lignans are bio-active compounds that are found in plants at different concentrations (Peterson *et al.*, 2010).

Plant tissue culture is a sterilized culture of cells, tissues, organs, and their elements, under defined *in vitro* conditions. It is a key instrument for both fundamental and applied research and for marketing purposes (Thorpe, 2007). Hairy root cultures are defined by fast development, genetic stability, and high-level secondary metabolite production produced when the plants genetically transformation of plants with *Agrobacterium rhizogenes*. Various bioreactor systems, including stirred tank, airlift, trickle bioreactor, rotating drum bioreactor, have been used to grow a hairy root. Some technical aspects were studied by hairy root cultivation in bioreactors (Liu *et al.*, 1999).

During the present study, a new model organism for toxicity testing *Caenorhabditis elegans* were being used (Figure 10). It is inexpensive, easy to grow on agar medium in petri dish maintained on a diet of *E. coli*, and well characterized. Among the invertebrates, it is considered as a unique organism for toxicological studies (Paul, 2011). *Caenorhabditis elegans* is a free-living nematode that is naturally found in soil of temperate climate and has become good model organism for parasitic nematodes research and a good testing system for possible anthelmintic compounds because it's cost-effective, readily available and easy to work with (Simpkina and Coles, 1981, Sant'anna *et al.*, 2013). The use of *C. elegans* to investigate the behavior, locomotion, reproduction, and death of nematode because it is simple and reliable (Thompson *et al.*, 1996).

Plant diversity and genotype analysis interactions were regularly carried out by morphological markers, but lately, different biochemical and molecular markers were established as strong instruments for such studies (Mahfouze and Ottai, 2011). Genetic diversity is different between the species in their genetic make-up and genetic variations (Julia, 1994). Natural pharmaceutical research is a key strategy for the discovery and development of new drugs. Nature and its numerous plant species represent a potential source since it contains innumerable quantities of molecules with a variety of pharmaceutical structures and activities (Mahiri, 2009).

Objectives of the project:

This study aimed to:

1. Establish Hairy Root cultures for both plant species using genetic transformation with *Agrobacterium rhizogenes*.
2. Use Bioreactor to examine the quantity of both plant secondary metabolite comparing with the fexed air culture.
3. Compare metabolic profiles of seeds, normal roots, and hairy roots.
4. Determine the genetic diversity within the varieties of *Hyoscyamus niger* and *Sesamum indicium* and establish the genetic relationship among the varieties.
5. Compare the metabolic diversity of the varieties with their genetic diversity.
6. Relate genetic and metabolic diversity in *Hyoscyamus niger* and *Sesamum indicium* to their geographic origin.
7. Establish a *Caenorhabditis elegans* model for bioassay of *Hyoscyamus niger* and *Sesamum indicium* metabolites and its volatile metabolites and product of thermal decomposition.
8. Test methanol extracts, Volatile metabolites, and thermal degradation product of *Hyoscyamus niger* and *Sesamum indicium* extracts in the *C. elegans* model for their anti-helminthic potential.
9. Carry out secondary metabolite profiling for selected varieties of *Hyoscyamus niger*, which is an important medicinal plant, and *Sesamum indicium*, which is known for health-promoting properties.

Advantages of the project:

1. Establishing a relationship between genetic and metabolic diversity in *H. niger* and *S. indicium* will help to design and screen strategies for high-producing genotypes of *H. niger* and *S. indicium* as a source of alkaloids and lignans for medical use.
2. Comparison of secondary metabolite production in seeds, roots, and hairy root cultures has been carried only with individual varieties so far; our study for the first time revealed to

which extent the difference in lignan content among plant tissues and hairy root cultures are genetically determined.

3. Toxic metabolites of *H. niger* so far are nonvolatile molecules. Comparison to the toxicity of volatile metabolites, thermal degradation release products, and soluble metabolites in a nematode model may shed light on the treatment of human eye infection by nematodes with *H. niger* smoke as used in indigenous medicine.
4. Comparison of lignan content in hairy roots and plants of *H. niger* and *S. indicium* for a set of well-characterized varieties represented the worldwide genetic diversity of the crop will relate genetic determinants responsible for stimulation of lignan production in hairy root cultures to the overall genome diversity of *S. indicum*.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Medicinal plants

During human history, medical plants were identified and used (Rachuonyo, 2016). Medicinal plants are almost universally used for the treatment of diseases in almost all the communities and often affordably more than costly conventional drugs (Fabricant and Farnsworth, 2001). Nowadays, millions of people worldwide use herbal medicines for several medical disorders as part of traditional medicine. The use of conventional medicine in underdeveloped nations directly contributes to the social, economic, and health status of rural communities (Tabuti *et al.*, 2003; Pattanaik *et al.*, 2006).

Herbalists and traditional medicinal workers, in particular, generate income from medicinal plants. The use of medicinal plants has become particularly important in primary health care (Organisation, 1995). In addition, some plants considered an important nutritional source and therefore recommended these plants for their therapeutic values (Rasool Hassan, 2012). The medicinal plant sections that can be used include various kinds of root, seeds, leaves, flowers, fruit, or all plant parts. Active compounds are directly or indirectly in almost all medicinal plants and are used as medicinal agents (Jamshidi-Kia *et al.*, 2018).

With the introduction of new and innovative technologies for the isolation and identification of natural products, the screening of new plant natural products has improved considerably. A library of fully-natural products can produce the best results that allow most scientists to quickly isolate leading compounds, such as full or partial drug synthesis, *in vivo* experiments, and clinical trials, to progress faster (Figure 1) (Sarker and Nahar, 2013, Jamshidi-Kia *et al.*, 2018). In modern medicine, over 120 active compounds are isolated until now from higher plants, and 80% of them show a positive relationship between their traditional and modern therapeutic uses of those plants (Fabricant and Farnsworth, 2001). World widely, traditional medicine was used to support, endorse, and restore human health (Sucher and Carles, 2008). For instance, traditional Chinese medicine is a fully-defined system that runs simultaneously with modern medicine that has been successfully used to diagnose, treat, and prevent diseases (Holtz, 2007).

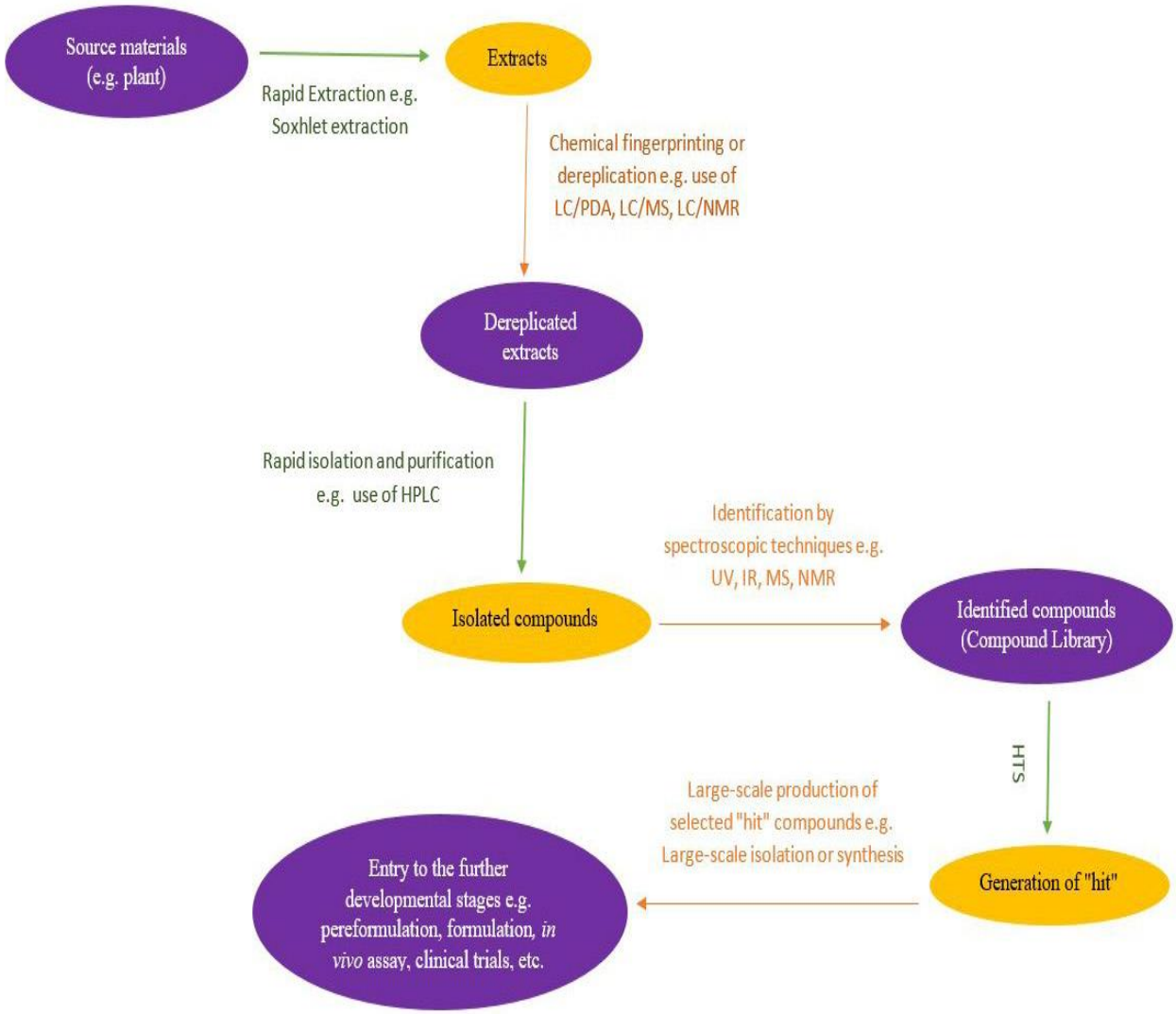

Figure 1: Modern discovery process of natural herbal remedies (Jamshidi-Kia *et al.*, 2018).

2.2. Black Henbane (*Hyoscyamus niger*)

2.2.1. Plant History and Description

Hyoscyamus niger considered a biennial or annual plant. It has a long flowering pattern, while it is treated with a cold duration, often combined with a photoperiodic system to initiate flowering (Raghavan and Wong, 1986). The leaves are oval and large with a greenish-grey color, and excessive hairy surface, grow from one to a half meters in length (Saidon, 2008), the flowers are almost without stalk and the yellowish glove-shaped corolla with purple veins. As the fruit grows, the calyx grows and surrounds the seed capsule (HeiserJr and Pickersgill, 1969). Approximately 96 genera with 3,000 species belong to the Solanaceae, commonly known as the Nightshade family. They are widespread in America, Europe, Asia, and Australia (Figure

2). Widely spread in temperate regions and grow in certain tropical regions with great difficulty (Sajeli *et al.*, 2006; CABI, 2019). A large number of economic, agricultural, and pharmaceutical plants are in this family (Wink, 2003). Solanaceae ranks among human beings as one of the most significant plant families, and they are very diverse. The Solanaceae plant's family is also rich in alkaloids, but only in fifteen genera of this wide family contained tropane alkaloids 6 of these genera, like *Atropa belladonna* and *Hyoscyamus niger*, are commercially significant (Saidon, 2008). The Solanaceae is a herbal dicotyledon family, and the term Solanaceae comes from the nightshade Latin (Solanuni) of the sun, so-called because some plants prefer to grow in shade area, and some flower at night (Stewart, 2013).

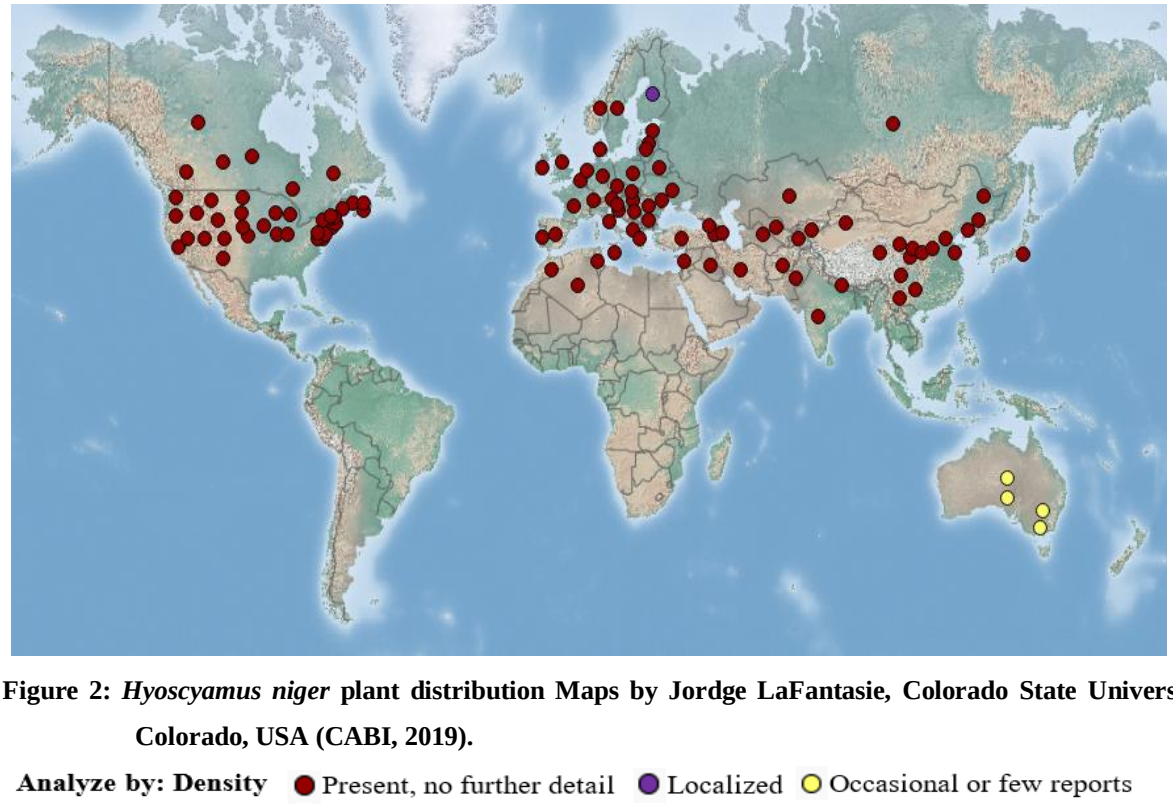

Figure 2: *Hyoscyamus niger* plant distribution Maps by Jordge LaFantasie, Colorado State University, Colorado, USA (CABI, 2019).

Black henbanes word is extracted from the Greek terms (hyos), hog and (kyaxnos), bean, and niger; Latin was used for black in reference to the seed color. From ancient Greece to present *Hyoscyamus* plant is used as a drug. Furthermore, the leaves and flowering plumage in the United States Pharmacopeia have been officially recorded from its first edition (Haas, 1995). The majority of species in the genera and at least three species, namely *Hyoscyamus, niger* (Figure 3), *muticus*, and *albus*, are medicinally important. And they are a good cultivated source of hyoscyamine, hyoscine, scopolamine, and atropine as the source of tropane alkaloids (Lavania *et al.*, 2010).

The name of the Henbane is taken from the Anglo-Saxon word, Henn (chicken), and Bana (assassin) because the fowls are paralyzed and cause death when the chicken eats the seeds. The whole plant is poisonous, and when eaten, small quantities, together with other anticholinergic effects, cause plenty from dizziness to delirium. In the Anglo-Saxon wording, "If a man cannot sleep, take the seed juice of henbane and garden mint, shake and strain his head; that is fine to him (Haas, 1995). Black Henbane has a little sticky hair, and it is annual or biennial. In the first year, there is a brooch of basal leaves, followed by a simple , upright, or slightly trimmed stone up to 80 cm high and covered with sticky hair in the second year (Ismeel, 2011).

Figure 3: *Hyoscyamus niger* (Taken by the Author, 2017).

The radial leaves are placed around the neck of the root-like a rosette. They are long, egg-shaped, and more than 30 cm long, sharp, and stalky with toothed edges. They are greenish-gray and covered with sticky hairs (Pudersell, 2006). The stems have a height between 20 and 100 cm, are erect and slender. The leaves are long, egg-shaped, pinnate, pulpy, and greyish-green with a stalk of bottom leaves and half of the upper amplexicaul. Flowers sit on the top of the stems in swirls. The bell-shaped crown is dirty yellow, with a reddish-violet throat and violet reticulated veins (Pudersell, 2006). It generates large quantities of seeds between 10,000 and a half million seeds per plant, and just 10-20 seeds are sufficient to poison a child. The fruit is an explosive capsule. Up to 500 gray-brown seeds exist in fruit capsules (Chevallire, 1996).

2.2.2. Classification

The plant classification was done according to (USDA and NRCS, 2019) as below:

Kingdom	Plantae
Subkingdom	Tracheobionta
Super division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Solanales
Family	Solanaceae
Genus	*Hyoscyamus* L.
Species	*Hyoscyamus niger* L.

2.2.3. Alkaloids

From ancient history, *Hyoscyamus niger* has been used for folk medicinal, religious, and ritual purposes. All the plant parts have a medicinal value, The chemical profiling and phytochemical studies of *Hyoscyamus niger* discovered the presence of a very important Alkaloid like Hyoscine, Atropine, scopolamine, hyoscyamine, anisodamine, coumarin, kaempferol, quercetin, rutin, cuscohygrine, linoliec acid, myristic acid, chlorogenic acid, oleic acid, stearic acid, pyridine, anisodine, trimethylamine, B-sitosterol, aesculetin, grossamide, cannabisin D & G daucosterol, vanillic acid, calystegines and anolides (Begum, 2010). Moreover, the new studies recorded the expectance of many important non-alkaloidal constituents, including lignanamides, withanolides (Ma *et al.*, 2002), flavonoids, and tyramine derivatives in *Hyoscyamus niger* L (Ma *et al.*, 1999). The earlier studies reported the existence of a lignan, hyosmin (Ajeli *et al.*, 2006), and four coumarinolignans: venkatasin, hyosgerin, and cleomiscosins A and B from the seeds of *H. niger*. The continued interest in the lignans of *H. niger* led to the isolation and characterization of two lignans, hyoscyamal and balanophonin, along with two glucosides, pongamoside C & pongamoside D which are new to the Solanaceae family (Sajeli *et al.*, 2009).

The term Alkaloids is derived linguistically from the word (al-qali), which was originally obtained from sodas, nitrogen compounds that are mainly, but not exclusively, the pharmacologically active "basic principles" of floral plants (Kutchan, 1995, Linhai Wang, 2014). Alkaloids are structurally different kinds of nitrogen compounds found in many plants, which have frequent physiological activity. Plants that produce alkaloids and extracts for their medicinal and toxically properties have always been used throughout history. Modern plant-

based alkaloids are commonly used in analgesics (morphine and codeine), in stimulants (caffeine and nicotine), and in chemotherapy (vincristine, vinblastine, camptothecin, and paclitaxel) (Hughes and Shanks, 2002). Many alkaloids showed to be toxic to other organisms and are frequently used as drugs, as a recreational drug or in the entheogenic rituals as raw extracts by acid extract-base mining.

Humans traditionally use alkaloid toxicity in hunting and fighting and are nowadays involved in treating diseases (Luca and Pierre, 2000). Nicotine is used as a powerful natural insecticide and a potential anti-inflammatory agent as a typical pyridine alkaloid (Isman, 2006, Mabley *et al.*, 2011), the more intensively studied tropane alkaloids are hyoscyamines hyoscine, and scopolamine's, probably due to their anticholinergic activity on the parasympathetic neurological system (Yun *et al.*, 1992). The treatments for motion sickness, cardiac conditions, and gastric disorders are cared out using hyoscyamine and scopolamine (Vazdekis *et al.*, 2008).

2.2.3.1. Lignans

The family lignan is considered as a large group of obviously plentiful molecules found in a large number of higher plants (Sainvitu *et al.*, 2012). Lignans from plants are bio-phenol compounds and were first found in wood tissue trees in the 19th century (Patel *et al.*, 2012), lignans considered as one of the main secondary metabolite of plants derived from the phenylpropanoid pathway, plays an important role in plant protection and are most effective in human food and medicine (Hazra, 2016). Lignans and neoligans represent a large group of phenolic compounds, which occur naturally, characterized by two C6C3 units being coupled. The compound is referred to as lignan when the two C6C3 units are linked by a link between 8 and 8 ' compound. If two C6C3 units are connected by other carbon bonds, they are called neolignans (Figure 4) (Moss, 2000, Parreiraa *et al.*, 2010).

The pharmacological and clinical interest in cancer treatment and other diseases is highly significant (Hemmati, 2007). In 70 plant families and a variety of localities, the Lignans were discovered to be found in roots, leaves, seed, and plant flowers (Pilkington, 2018). Most importantly, there were several powerful, significant biological activities in this class, including anticancer activities (Pan *et al.*, 2009), antimicrobial (Saleem *et al.*, 2005), antiviral (Yousefzadi *et al.*, 2010), immunosuppressive, anti-inflammatory (Saleem *et al.*, 2005), antioxidant (Pan *et al.*, 2009), and hepaprotective (Huang *et al.*, 2010), actions as well as cancer (Webb and McCullough, 2005) and osteoporosis (Habauzit and Horcajada, 2007) activities that ledes to growing interest in the synthesis of lignans (Pilkington *et al.*, 2015). Lignans such as

podophyllotoxin and α-peltetin have revealed some antiviral activity besides cytotoxin. Some lignans are also recognized for anti-hepatotoxic, anti-HIV, antimicrobial, and anti-depressant activity (Hazra, 2016).

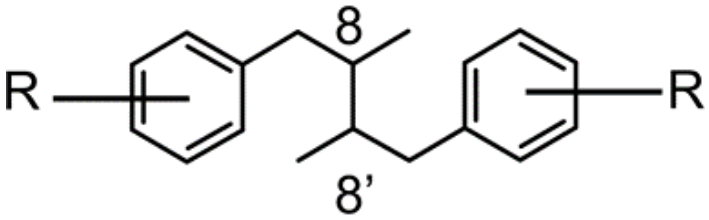

Figure 4: Lignans structure. Between 8 and 8 '' carbon atoms, two C6C3 units are connected (Moss, 2000).

2.2.4. Medicinal importance

From ancient history, the uses of *Hyoscyamus niger* for medicinal, religious, and ceremonial purposes were recorded (Saidon, 2008). All parts of the plant are with medicinal values. *H. niger* is used in many ways as a sedative, pain killer, and specifically for urinary-tract pain, especially when due to kidneys stones. They have a very long history as a medicinal herb. For its antispasmodic action, hyoscine and hyoscyamine that were previously identified in *H. niger* as anticholinergic constituents are considered to be responsible, however, the plant usually contains several compounds operating at different locations (Gilani *et al.*, 2008).

The ancient Egyptians inhaled the heated vapor of *Hyoscyamus muticus* containing antimuscarinic alkaloids; scopolamine for asthma-treatment (Barnes, 2006), Alkaloid in *H. muticus* effects stimulus for parasympathomimetic, spasmolytic, anticholinergic, narcotic, and anesthetic properties of the central nervous system simultaneous depression of the peripheral nerve (Ibrahim *et al.*, 2009).

The *H. niger* seed is used as an anthelmintic antitumor in Tibetan medicine. They are found to be suitable for treating intestinal/stomach pain caused by worm infestations, toothache, pulmonary region infections, and tumors (Begum, 2010). Although it is known as a poison plant in Indian and Chinese medicine, it is traditionally used for stomach cramps, the heaviness of tobacco, neuralgia, and manic psychosis. Furthermore, the seed oil is used extremely for dental, rheumatic, and neuralgic pains (Begum, 2010). Henbane was also used to treat rabies, fever-related delirium, bronchitis, renal coughs (Harrison and Bartels, 2006). *H. niger* methanol extract has the anticonvulsants against seizures caused by picrotoxin in the mouse (Heidari Mahmoud Reza, 2009).

2.3. Sesame (*Sesamum indicum*)

2.3.1. Plant Description and Importance

Sesame (*Sesamum indicum* L.) is a very important plant cultivated in Asia, Africa, South and Central America's tropical and sub-tropical regions (Figure 5 and 6), as states by the (FAO) Food and Agriculture Organization of the United Nations (Rehana Naz, 2011, Fuji *et al.*, 2018). As described in ancient texts such as the vedas, sesame is also conceded as one of the herbal medicines and healthcare preparations, it has a religious and mythical significance and is used in villages for sacrifices and ceremonies of marriage. In Asia, sesame has been cultivated for more than five thousand years and is among the oldest crops in the world (Bisht *et al.*, 1998, Deogade and Pandya, 2013).

The origin of Sesame has been a topic of debate among hundreds of writers for more than a hundred years. In Africa, many invoke domestication based on geographical affinities, a common claim that is untested (Bedigian, 2003, Bedigian, 2010). Furthermore, on the other side, there is proof that the Indian subcontinent is domesticated on the grounds depending on the formation of fully fertile hybrids, lignan components, and RAPD analysis (Bedigian, 2004).

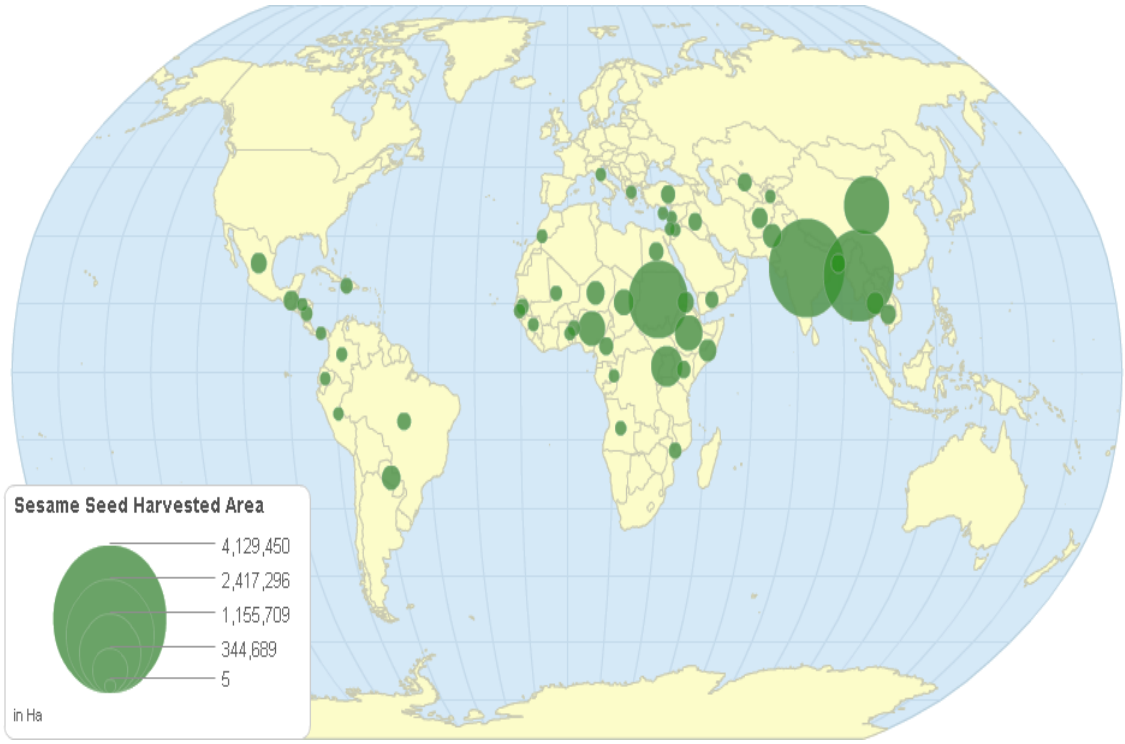

Figure 5: *Sesamum indicum* plant distribution Maps, by ChartsBin.com, from Food and Agriculture Organization data, (ChartsBin.com, 2019)

The largest sesame producers in the world are India and China today, then followed by Myanmar, Sudan, Uganda, Pakistan, Nigeria, Tanzania, Ethiopia, Guatemala, and Turkey (Figure 5) (Deogade and Pandya, 2013).

Sesame is an annual plant with a length of between 50 and 100 cm. It is made of up to 14 cm opposite leaves with a complete margin; lances are wide and 5 cm wide, at the base, and are reduced in width on the flowering stem to only 1 cm wide. Flowers are yellow and tubular, with a four-lobed mouth and a length of between 3 and 5 cm. The flowers may be white, blue, or purple (Bedigian, 2010). Approximately 36 species are known to exist. Mainly in Africa, with just a few in India, it has been mentioned that Ethiopia may be the origin of this plant (Bisht *et al.*, 1998).

Figure 6: *Sesamum indicum* L. (By the Author, 2017).

In comparison to many major oil crops, sesame oil has relatively high quality. The oil content is 34.4% to 59.8%, but in most cases is approximately 50% of the seed weight. In some varieties, the value of up to 63.2% was reported (Were, 2006). Sesame oil contents affected by genetic and environmental factors. It is reported that late mature cultivars have higher oil content than earlier cultivars (Yermanos *et al.*, 1972).

2.3.2. Classification

The plant classification was done according to (USDA and NRCS, 2019) as below:

Kingdom	Plantae
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Asteranae
Order	Lamiales
Family	Pedaliaceae
Genus	Sesamum L.
Species	*Sesamum indicum* L.

2.3.3. Active Ingredients

Sesame plant products act as antimicrobial agents. It contains several categories of useful antimicrobial compounds: polypeptides, phenolics, essential oils, alkaloids, terpenoids, and lecitins (Cowa, 1999). Sesame also contains several important secondary metabolites and active ingredients like sesamol, sesamolin, sesamin, sesaminol, chlorosesamone A, B, C, D, and E, hydroxysesamone, anthrasesamone, 2,3-epoxysesamone, and anthraquinone pinoresinol (Srivastava *et al.*, 2001).

The largest oil-soluble sesame lignans were considered to be Sesamin and sesamolin (Namiki, 2009), while sesame also contains piperitol, sesamolinol, pinoresinol, sesaminol, episesaminone, allohydroxymatairesinol, hydroxymatairesinol, and larisiresinol, which have free phenolic groups and consequently antioxidant activity in sesame seeds has been reported.

The wild species of sesame also contain sesamolin and angustifolium and 2-episesalatin (Osawa *et al.*, 1985, Kamal-Eldin *et al.*, 1992). According to Chinese Materia Medica, the Japanese Edition, the *S. indicum* contains 0.3 percent of the polyphenol pedaliin (pedalitin-6-O-glucoside; 6-hydroxy-luteolin7-methyl ether 6-glucoside), without citing any source (Fuji *et al.*, 2018). According to Naokata Morita, the pedaliin existence in sesame plant based on its recrystallization from methanol extract (Morita, 1960), also pedaliin has been detected in cultured sesame tissue (Jain, 1981). The oil's fatty acids consist mainly of oleic and linoleic acids, but have only trace amounts of linolenic acid, with small amounts of palmitic and stearic acid. Linoleic, linolenic, and arachidonic acids are considered essential in nutritional terms, although linoleic acid is presumed to be in vivo synthesized from linoleic acid (Namiki, 2009). Sesame seeds are one of the good sources of calcium, phosphor, and iron. Half to two-thirds of

calcium is present in the seed as oxalate, with a large number in the test. Seeds are usually used after dehulling, so oxalate does not interfere with calcium absorption, Sesame seeds have high thiamine, riboflavin, and nicotinic acid levels (Bedigian, 2003).

2.3.4. Medicinal and economic importance

Sesamum indicum L is an ancient kernel oil commonly used as a food and medicine source (Hsiao *et al.*, 2006). In Africa and Asia, sesame is well-known in folk medicine. Most of the plant parts are useful. The decoction of the leaves is, however, used to treat bruised or erupted skins, colds, and eye aches in south-western Nigeria. Hot water Infusion of the leaves is used for gargling inflamed mucosal membranes. The decoction of both leaves and roots shows antiviral activity, and also found to be active against measles and chickenpox and used as a hair shampoo for Taenia capitis (anti-capitulation) (Munir *et al.*, 2007).

Sesame seeds contain polar, nonpolar, and semi-polar compounds, the seeds may have different polarities, resulting in different antioxidant capacities (Ruslan *et al.*, 2018). The sesame seed oil has been used for thousands of years for healing and has been enjoyed by people since civilization began (Laj *et al.*, 2007).

Sesame is very beneficial for loose teeth, pyorrhoea and toothache Dental problem, Bleeding dysentery, Burn, Impotence, Menstrual cycle Sesame consumption can be very helpful in the event of severe menstrual or menstrual pain (Deogade and Pandya, 2013). Anti-inflammatory activity and antipyretic: The antipyretic effect of sesame oil was significant in comparison to paracetamol. In previous studies, the formation of pleural exudate and leucocyte migration was inhibited by sesame oil and sesame and confirmation of the anti-inflammation activity (Monteiro *et al.*, 2014).

Anti-oxidant effect of *S. indicum*: Sesame increases vitamin E recycling, enhances the functioning of the liver and protects against oxidative stress due to alcohol. Sesame reduces cholesterol while increasing lipoprotein levels with high density (Ide *et al.*, 2003). Sesame oil improves the hepatic detoxification of chemical, decreases the incidence and oxidative stress of chemical-induced mammary tumors, which contribute to the pathogenesis of endotoxin intoxication (Yasumoto *et al.*, 2001, Shasmitha, 2015).

Anti-microbial activity: Naturally, sesame is antibacterial to common skin pathogens, like streptococcus and staphylococcus, as well as to skin fungi, like foot fungus of an athlete. Sesame oil has the best antimicrobial action as well as the highest area of inhibition against *Salmonella Typhi*, equivalent to the standard Kanamycin. It has been found to be antibacterial for *Streptococcus mutans, Lactobacilli acidophilus* (Durai Anand *et al.*, 2008).

Anti-hypertensive activities: Sesame oil intake has significantly reduced oxidative stress and increases GPx, super oxidase dismutase, and catalase activities in hypertensive patients. The findings support the assumption that the consumption of sesame oil could help to improve the antioxidant defense system in humans (Sankar *et al.*, 2005, Shasmitha, 2015). Wound healing properties: At the site of the injury, free radicals are generated that are known to damage cell membranes, proteins, and lipids. Powerful antioxidant activity of sesame oil extract can help to prevent oxidative damage and promote healing (Fukudaa *et al.*, 1986). Anti-cancer properties: the proliferation of human colon cells and the growth of malignant melanoma in vitro have been found to be inhibited by sesame oil (Edwards Smith and Salerno, 1992).

2.4. Plant Tissue Culture

In lead discovery, natural products play an important role, particularly in oncology, cardiovascular, epidemiology, and metabolic diseases. Today, the amount and variety of phytochemicals used by society as biopesticides, nutrients, and in particular cosmetic additives are continually expanding, and research efforts to create new forms of producing metabolites from plant species are driving these high demands (Sena, 2015). The tissue and plant cell culture techniques constitute valuable alternative systems to produce innovative metabolites, particularly as plant cells (Figure 7), tissues and organs can be cultivated in sterile conditions completely independent of geographical and climatic factors through the systems, thus developing various technologies based upon them (Murthy *et al.*, 2014).

2.4.1. Hairy root cultures: background and establishment

Plant tissue culture has been considered an alternate source of biologically active compounds over many years (Łuczkiewicz and Kokotkiewicz, 2005). From the beginning of the last century, two types of plant diseases, known as hair root and crown-gall, caused substantial losses in the winegrowers, and several investigations into their nature and the infection mechanisms involved were initiated (Sena, 2015).

The hairy root is a disease infects the dicotyledon plant caused by the Gram-negative soil bacterium *Agrobacterium rhizogenes* (Sevón and Oksman-Caldentey, 2002). When the plant been infected by the bacteria, the T-DNA of the Ri-plasmid in the bacterium is transported into the infected plant's nuclear genome. A valuable by-product will produce, which called hairy root (Figure 8), (Chilton *et al.*, 1982, Georgiev *et al.*, 2007). Hairy roots are rapidly growing and are vastly branched structures produced on phytohormone-free medium. It is highly differentiated with genetically and biochemically stable structures that can cause constant and

wide-ranging secondary metabolites production, whereas the other plant cell cultures did not have the same positive specifications (Kittipongpatana *et al.,* 1998).

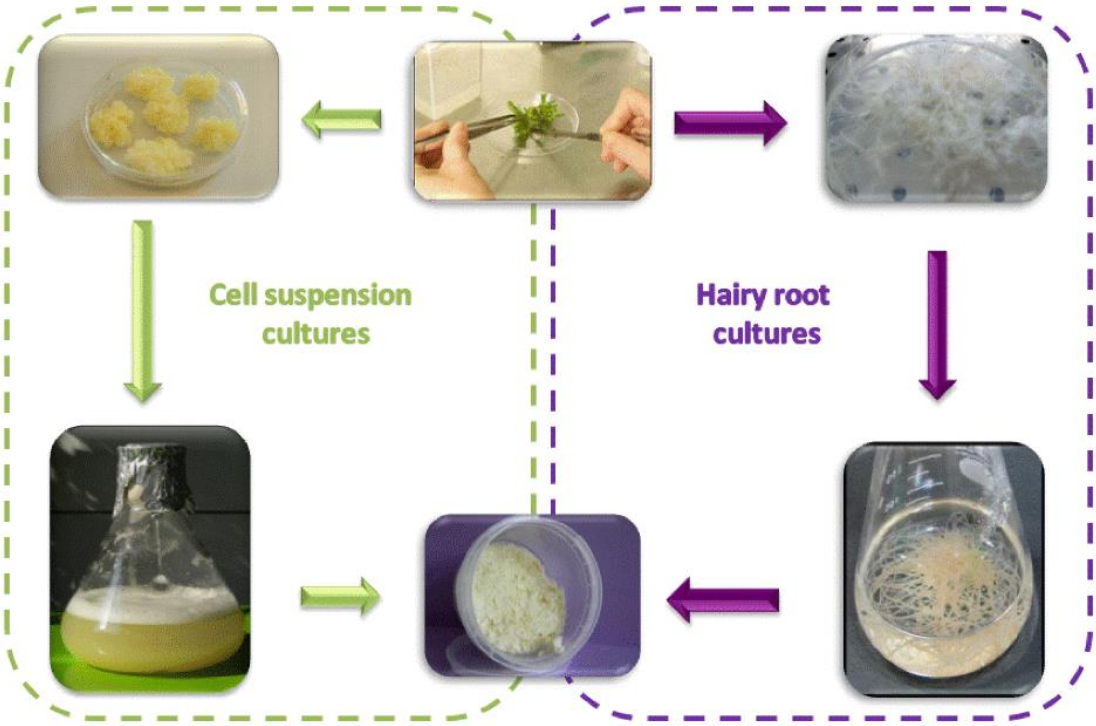

Figure 7: Plant tissue cultures techniques. Plant cell cultures are a valuable source of plant important secondary metabolite, but hairy root culture is a revolutionary alternative production procedure (Source: (Sena, 2015)).

In biotechnology, this natural occurrence is used to produce hairy root cultures. The induction procedure for hairy roots comprises the culture of the injured plant part (explants) with *A. rhizogenes* suspensions in aseptic conditions (Georgiev *et al.*, 2007). Several essential conditions should be taken into account for establishing a successful hairy root culture system. These conditions include *A-rhizogen* bacterial strain, an appropriate antibiotic, and a proper culture media for eliminating redundant bacteria after cultivation (Hu and Du, 2006). The advantage of plant cell suspension culture is rapidly growing and independent from geographic and seasonal differences. Plant suspension cells remain undifferentiated during cultivation, whereas secondary metabolite biosynthesis is often ineffective and unstable among undifferentiated cells (Zhao, 2014).

Hairy root culture has many advantages in comparison to undifferentiated cell suspension cultures (Flores *et al.*, 1999). Hair roots grow in hormone-free media and are genetically stable and thrive. Hairy roots show fast growth and encourage phytochemical synthesis that requires different cell types in the biosynthesis (Dehghan *et al.*, 2012). Some instances of accumulated new metabolites were found in transformed roots cultures, such as two new isoprenylated

flavonoids, and novel benzoquinone hydroxy chinofuran B in *Lithospermum erythrorhizon* (Eapen and Mitra, 2001).

For the first time in 1987, Flores established the hairy root cultures of *H. niger*. They reported that hairy roots produce tropane alkaloids, often similar to or larger than those in intact plants (Kokotkiewicz, 2005). Hairy root cultures of *Hyoscyamus muticus* have, therefore, been considered as a promising protocol for *in vitro* tropane alkaloid production from that time (Zolala *et al.*, 2007). Hairy root extracts from *S. indicum* showed antimicrobial activity much higher than that of the mother plants. The metabolite content of the hairy roots was, therefore, expected to be higher than that of the mother plant (Ogasawara *et al.*, 1993).

2.4.2. Agrobacterium-mediated transformation

Agrobacterium rhizogenes is a part of the soil bacterial family *Rhizobiacae* and responsible for a wide variety of dicotyledon plants and few gymnosperms hairy root disease (Eapen and Mitra, 2001). Hairy roots are formed by plant infection with *Agrobacterium rhizogen*, a gram-negative bacterium of the soil containing a (Ri) plasmid for root-inducing (Huffman *et al.*, 1984) (Figure 8).

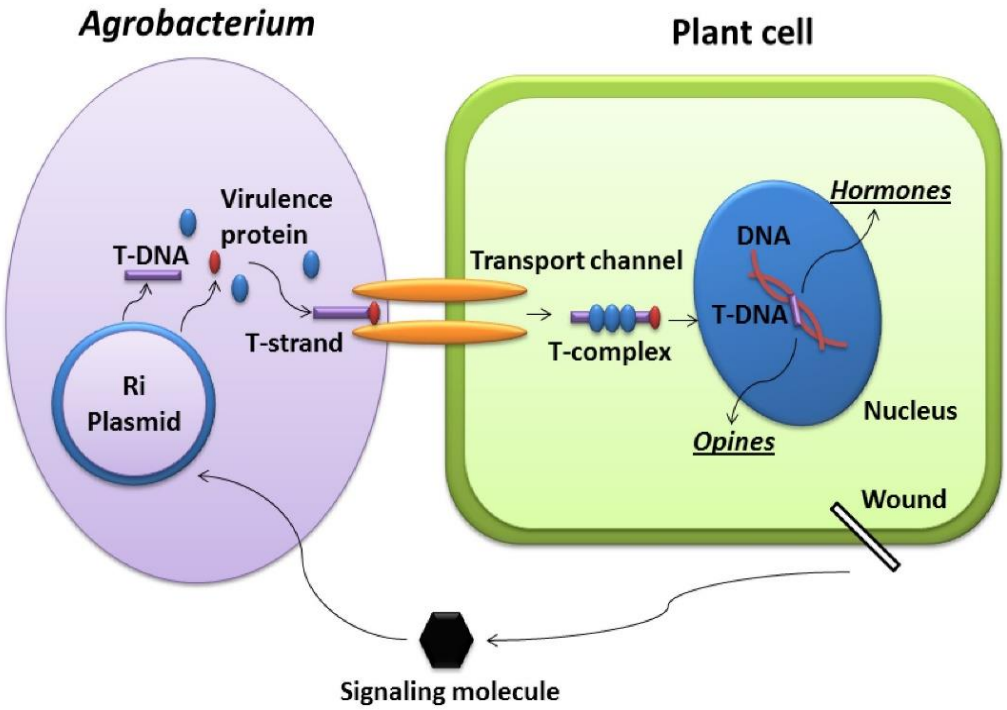

Figure 8: *Agrobacterium* mediated transformation plant cell (Source: (Zhao, 2014, Gelvin, 2005)).

2.4.3. *Agrobacterium rhizogenes* ATCC 15834

The ATCC 15834 strain of *Agrobacterium rhizogen* has been obtained from ATCC for the hairy root culture of tomato (Ron *et al.*, 2014). The bacteria were strained on solid medium and cultivated at 28°C for two days (Wis´niewska *et al.*, 2013). Strain cultures were cultivated with a liquid medium for electrocompetent cell preparation and extraction of DNA (Ron *et al.*, 2014, Casimiro *et al.*, 2001). The ATCC 15834 *A. rhizogen* strain contains 6,919 coding and 54 RNAs. Predicted sequences, from this annotation, a full length (1,416 bp) 16S sequence was obtained and used to identified rhizobium sequences compared with 54 publicly available Agrobacterium 16S sequences by MUSCLE alignment (Edgar, 2004, Ron *et al.*, 2014). Which was used to build a Fast Tree two phylogenetic tree (Price *et al.*, 2010). In many of biotechnologies and research applications, *A. rhizogenes* ATCC 15834 was used to induce hairy root culture (Ono and Tia, 2011, Ron *et al.*, 2014).

2.4.4. *Agrobacterium rhizogenes* A4

A4 strain of *Agrobacterium rhizogenes* is a virulent type Agrobacterium that has three plasmids: for plant transformation, a (pArA4a, 180 kb) plasmid is not necessary. b (250 kb) is a plasmid that induces root (pRiA4), and (pArA4c) plasmid is a (pArA4a) and (pRiA4) cointegrate (Jouanin *et al.*, 1986). The *A. rhizogenes* agropine-type strain A4 transfers two distinct segments of T-DNA into plant cells (Taylor *et al.*, 1985). The T-DNA on the right (TR- DNA) has a Ti plasmid T-DNA homology involved in auxin biosynthesis and the production of agropines, while the left-hand T-DNA (TL-DNA) is homologous to an undefined region near the left edge of T-DNA type nopaline (White and Nester, 1980). The comparison of the plasmid restraint maps of strain A4 suggests that the recombination event for pArA4c formation takes place in the same regions of pArA4a and pRiA4. Furthermore, a comparing with the already established pRiHRI map shows that strain HRI can be derived by percentage points from a recombination event between the two homologous pArA4c areas with subsequent loss of the smaller plasmid (Jouanin *et al.*, 1986). Recent studies have shown that Ri plasmid homology comprises genes that affect the formation of the tumors and morphology (White *et al.*, 1985, Taylor *et al.*, 1985).

2.4.5. Yield Enhancement Strategies

The primary reason why bioactive compounds use HRCs are not produced commercially is their low production yield; this leads to high costs of molecule production. Several strategies were used to make hairy root technology viable to the greatest possible extent. The preliminary

strategy in this respect is the optimization of the growth medium. In general, hairy roots can initiate on any base media used in the practice of tissue culture, but the nutritional requirements vary with both the plant system and the objective of establishing the roots (Mehrotra *et al.*, 2015). Bioreactor technology has been developed to promote large-scale setups for hairy root growth and metabolite production, and companies willing to cultivate hair-root biomass for the production of metabolites (Kim *et al.*, 2002).

2.4.5.1. Bioreactors for hairy root cultures

The bioreactor is an apparatus, such as a large vessel, for the cultivation of any plant parts, which are attractive expression systems for the economical production of pharmaceuticals compounds (Figure 9). Bioreactors can be used for the production of biotechnological ingredients such as active plant substances, antibodies, or vaccines or in the bio-conversion of organic waste (Mishra and Ranjan, 2008).

Bioreactor technology can be conceived as a strategy for improving yields. Previous studies on hairy roots growing in the bioreactor, keep up the idea of designing a cultural vessel to facilitate the supply of oxygen nutrients that may result in tissue growth improvement or hairy root crops (Srivastava and Srivastava, 2007, Mehrotra *et al.*, 2015). There are three types of bioreactors: liquid-phase bioreactors, gas-phase bioreactors, and both combinations. The bioreactors in the liquid phase, the hairy roots, are immersed in the medium, hairy roots are exposed to gas in the gas phase, and nutrients are supplied with droplets in hairy roots (Kim *et al.*, 2002). The bioreactor can be divided into a batch culture, continuous culture, and semi-continuous culture on the base of the mode of operation. The choice of bioreactor operation for cultivating the hairy root is determined by different purposes (Mehrotra *et al.*, 2015).

Liquid-phase reactors; Hairy roots are immersed in the liquid medium, and the term ' submerged reactors ' is also used (Kim *et al.*, 2002). Gas-phase reactors; the hairy roots are immobilized by using flat sheets or inert materials into cultural vessels and exposed to the mixture of air or gas. The fluid medium is supplied by spray or nebulae. Such an arrangement reduces much of the liquid reactor limitation (Flores and Curtis, 1992, Mehrotra *et al.*, 2015).

Hybrid bioreactors that are a combination of both liquid-phase, gas-phase reactors the growth and productivity of hairy root cultures are reviewed with prominence on successful bioreactors and important cultural considerations. Thy includes strain selection, production of secondary metabolites in relation to the growth phase, media composition, the gas regime, use of elicitors, the role of light, and apparent product loss (Williams and Doran, 2000, Kim *et al.*, 2002). The key issue in bioreactor design and work is to control the biochemical phenomenon in a

consistently optimized way for a defined period in order to obtain continuous highest productivity. This can be optimized on two levels: firstly, the biological entity and its product, including the physiological basis for the cell line, clone selection, synthesis of the metabolites, and accumulation. The second optimization scale comprises physical parameters of culture vessel such as air supply, temperature, pH, removal of the product, medium continuity, (Kim *et al.*, 2002, Srivastava and Srivastava, 2007).

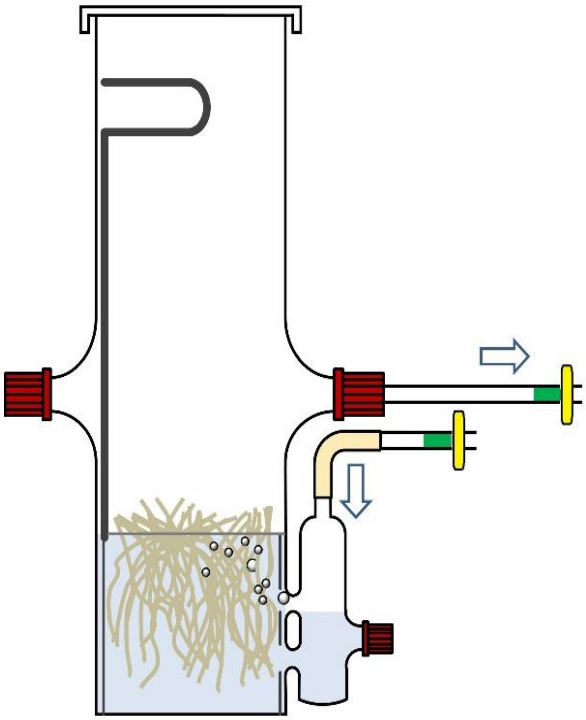

Figure 9: Schematic presentation of promising newly designed bioreactor configuration. (Source: The Author and Lng Su, 2018).

2.5. The biomedical activity of medicinal plants

Plant materials have been screened for pharmaceutical usage in traditional methods. If there is any activity evidence, the extract is fragmented, and the active compound is isolated and recognized (Kim *et al.*, 2002, Jamshidi-Kia *et al.*, 2018). There's a hopeful future of medicinal herbs as approximately half a million plants in the world are not yet studied, and current and future medical activity studies can be effective in treating diseases (Singh, 2015). For therapeutic purposes, it is possible to utilize different parts of medicinal plants, such as root, leaf, fruit, seeds, skin, flowers, or even the whole plant. In most medicinal plants, the active compounds are directly or indirectly therapeutic and used as medicinal products (Phillipson, 2001, Jamshidi-Kia *et al.*, 2018).

2.5.1. Anti Nematocidal Activity

During the history in many parts of the world, the medicinal plants have been used to fight against parasitism and other human and veterinary illnesses (Muthee, 2018). Helminth infections caused by trematode (flukes), nematodes (roundworms), and cestodes (tapeworms) affect humans and animals. They are of immense significance or human, veterinary medicine in the tropical region (Azim and Sajid, 2009). Nematode parasites (roundworms) cause serious morbidity to people, mainly in developing countries. Several nematodes caused diseases to humans, such as Filariasis, Strongyloidiasis, River blindness, *etc* (Hotez *et al.*, 2007). Around 1.5 billion individuals worldwide are infected with soil-borne helminths (STHs), with the majority of the world's poorest individuals. Human development, nutrition, cognition, school attendance and pregnancy, manufacturing, and income are being damaged by this disease (Hotez *et al.,* 2007). Helminthiasis is a severe global problem with elevated human disease and massive animal losses in tropical and subtropical nations in particular (Wailer, 1999). Anthelmintic treatments are used in the management of helminthic parasite diseases, newer and efficient anthelmintic are very important since the chemical drugs currently used in helminth control are costly, and mostly lose their efficacy in the next 20 years because of resistance problems (Mehlhorn *et al.*, 2011, Sant'anna *et al.,* 2013).

2.5.2. Antiparasitic activity of *Hyoscyamus niger* and *Sesamum indicum*

Hyoscyamus niger L. was traditionally used for thousands of years as a sedative and pain killer. It was part of the brake for the magic power of witches. Some data are available on using of Henbane for the treatment of earaches, sinusitis, dentures, headache, wounds, eye itching, stomatitis, insomnia, cough, asthma, bronchitis, mucus, severe menstrual bleeding, gout, neuralgia, sciatica, rheumatism, lip removal and folk use for oral and dental hygiene (Turker *et al.*, 2018). Although recorded as toxic plants, *H. niger* is traditionally used for use with stomach cramps, heavy taxes, neuralgia, and manic psychosis in Indian as well as in Chinese medicine. The plant also has anti-spasmodic, sedative, and analgesic characteristics (Duke *et al.*, 2002).
In Tibetan medicine, *H. niger* plants were used as anthelmintic antitumor and febrifugal. They are also shown to be helpful for treating stomach/intestinal pain because of worm infestation, toothache, lung region, and tumor infection. It is widely used in the urinary body as a pain killer, particularly when it has a renal stone. The plant oil is used externally for neuralgic, dentistry, and rheumatic purposes (Begum, 2010, Al-Snafi, Tsewang, 1994). Powdered *Hyoscyamus niger* seeds water extract, methanol extract, studied to have mixed anti-nematode infection (Akhtar *et al.,* 2000).

Comprehensively few selected herbs were describing as antiparasitic plants, from which *Moringa oleifera* and *H. niger* potential as an emerging candidate to phytochemically control parasites. The plant's kingdom is the earliest recognized opulent pool of medicinal antibacterial, anthelmintic, and insecticides (Fatima *et al.*, 2014). Methanolic and aqueous extracts of Henbane seeds and Moringa roots powdered have been shown to decrease EPG in sheep with a combination of nematode infection (Hussain, 2008, Mandal *et al.*, 2015). The methanolic and aqueous extract from a raw product at various concentrations has been screened to determine the place and moment of paralysis and killing worms. *Piperazine citrate* was used as a standard nematocidal compound, and it was found that the (PHFEE) polyherbal formulation ethanolic extract activity was higher than the (PHFAE) polyherbal formulation aqueous extract (Chaturvedi *et al.*, 2009).

Malaria is an infectious disease of the genus Plasmodium parasite, which includes fever and chills, anemia, and splenomegaly. One of the cheapest and easiest techniques for preventing this illness is to remove the vector generally made by insecticides and chemical pesticides, but nowadays it is attempting to use natural toxins and crop compounds to avoid them owing to the harmful effects of poisonous chemicals (Behravan *et al.*, 2017).

Nowadays, Sesame oil has mostly been employed in cosmetic, medical, and proprietary branded oils and medicinal products (SHAH, 2013). During the year of 2004 and 2005, two tests were performed to explore the response of three chosen cultivars of *sesamum indium* to three population densities in a *Meloidogyne incognita* root-knot (root-knot nematode). A new technique to assess and report *Meloidogyne spp.* resistance method was performed (Atungwu *et al.*, 2008).

Despite *Meloidogynejavanica's* broad host range, an appropriate crop cover or rotation may sometimes control it. *Crotalariajuncea* L. *Sesamum indicum* L and *Dolichos lablab* L, for instance, reduce the number of root-knots in soil nematodes (Araya and Caswell-Chen, 1994).

The antiparasitic characteristics of Sesame (*Sesamum Indicum* L., *Pedaliaceae*) oils and their constituents against microorganisms and insects have been examined in the latest years (Thattakudian, 2011). Several studies were conducted to evaluate the anthelmintic activity of methanolic extract of sesame seeds on aquarium worm Tubifex (Kamal *et al.*, 2015). The pharmacological characteristics of sesame lignans include antioxidant activity, anti-proliferative activity, reducing cholesterol concentrations, increased oxidation resistance of hepatic fatty acids, and antihypertensive impacts. The methanol extract from *S. indicum* plants, based on prior study findings, had excellent antiparasitic and antioxidant actions owing to the existence of phenolic content such as lignans and further subsequent metabolites (Yokota *et al.*, 2007, Nakano *et al.*, 2008, Nigam *et al.*, 2014).

2.5.3. *Caenorhabditis elegans*

The multi-cellular organism *Caenorhabditis elegans* is a member of the Rhabditidae family in the Rhabditida order of the class Secernentea, from the Nematoda phylum. (Oluwadare, 2017). *C. elegans* is a natural free-living nematode discovered in moderate-climate soils, is a model for the studies of nematode parasites and an outstanding tool for testing prospective anthelmintic compounds because it is cheap, easily accessible and simple to handle (Katiki *et al.*, 2011, Sant'anna *et al.*, 2013).

The *C. elegans* adults are approximately 1 mm in duration and 80 μm in diameter. Animals can be cultivated with *Escherichia coli* in the laboratory either in liquid or in agar plates and can be readily cultivated in high quantity (Figure 10). *C. elegans* have five autosome twins and one sex chromosome pair, the hermaphrodite pairing of the male population is easy to maintain. The hermaphrodite produces some 300 progenies through the self-fertilization process and more if it matches males (Palikaras and Tavernarakis, 2013).

C. elegans are either males, or self-fertilizing hermaphrodites need 2-4 days for reproduction, 2–3 weeks for existence, and can be deposited in fluid oxygen almost permanently. The nematode is clear and allows live-cell imaging (Elkabti *et al.*, 2018). The *C. elegans* nematode has already been used as an ecotoxicology biosensor. Using this model has several benefits: it is a multicellular organism, self-fertilization has a short life cycle, high progeny, and easily maintained on a micro-titer plate in the laboratory (Dengg and van Meel, 2004).

C. elegans is an outstanding method for testing components with potentially anthelmint activities and a model organism for mosquito nematode studies because it is cheap, easily accessible, and easy-to-work. Moreover, *C. elegans* are uncomplicated and safe when testing nematode conduct, locomotion, breeding, and mortality (Sant'Anna *et al.*, 2017). *C. elegans* ' drug screening offers many interesting alternatives. There are a series of models that depict varied human diseases, which not only help us search for fresh medicines but also offer us an idea of their metabolism and intervention systems in a complicated eukaryotic organism (Kadlecová, 2014).

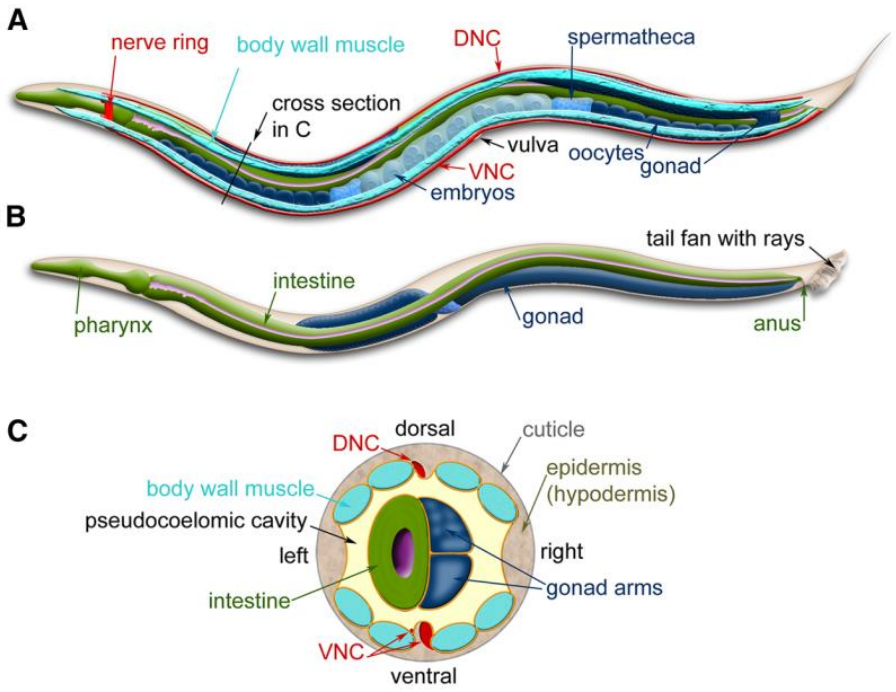

Figure 10: *Caenorhabditis elegans* anatomy. *Caenorhabditis elegans* anatomy. Major hermaphrodite anatomical characteristics (A) and male (B) viewed laterally. (Z. F. *et al.*, 2015).

2.5.4. Thermal released VOCs *Hyoscyamus n*iger

Vapor decontamination, consisting of the implementation of a vapor (or gas) decontaminant agent in confined spaces and medical devices, is amongst the approaches established to decrease the microbial pressure on the environment through biocides (Kačer *et al.*, 2012). It is very crucial in the use of the decontamination technique that effectiveness relies on the command of the following parameters: quantity of biocidal, exposure moment, heat, moisture, and contaminant circumstances. Decontamination and sterilization procedures in the pharmaceutical industry, machinery cleaning, health centers cleaning, and food products were implemented in Vapor phases (Bueno, 2015).

Several analyses have also verified that in vapor stages, the EOs used for thyme, citrus oils, eucalyptus globulus, melaleuca alternifolia, and lemongrass have a more powerful type of antibiotics (Nadjib *et al.*, 2014). Currently, although vapor screening platforms have been described for EOs, standard tests are not available for assessing vaporized products antimicrobial active activity (Yousef, 2014).

The anti-nematode effect of these compounds was enhanced by the duration of thermal treatment. In fact, it influenced the vapor pressure of the molecules, which can easily interact with microbial membranes when they are in gaseous form (Belletti *et al.*, 2007). The results

show that they are very active in inhibiting bacterial growth in very low concentrations, making them ideal for the progress of vaporized biocidal products (Belletti *et al.*, 2007). In the microbial cell, shrinkage and incomplete degradation between the modes of activity of these vapors have been discovered through a scan electron microscopy/atomic force microscopy of *Calbicans* exposed to *Cymbopogon* oil in the vapor phase (Tyagi and Malik, 2010, Bueno, 2015).

Excavations on an Ottoman ventilated oven or tandır show that the remnant of Henbane charred seeds is indicative of the use of *H. niger* seed smoke for medicinal fumigation. Henbane smoke, depending on the archeological proof from the Ottoman Empire, was practiced in Asia as traditional therapy for toothaches and other diseases (Mohagheghzadeh *et al.*, 2006, Tabata *et al.*, 2008, Fenwick and Omura, 2015).

2.6. Chemical Profiling of medicinal plant

The active ingredients are isolated, purified, and analyzed in order to obtain real compounds using different methods, such as High-Performance Liquid Chromatography and Thin Layer Chromatography. In addition to this, the chemical analysis of therapeutic compound different analysis protocol card out like spectroscopic methods Liquid Chromatography, Liquid Chromatography-Mass Spectrometry, Nuclear Magnetic Resonance, Gas Mass Spectrometer, Infra-red and Ultra Violet / Visible Spectroscopy (Mngeni, 2017).

2.6.1. Phytochemical Studies

Phytochemistry can be articulated as a connection between chemistry and biology. Phytochemistry concerns the research and description of the chemical compound from multiple crop processes, and the chemical structure of products or natural compounds and the practice of the phytochemicals within the living organisms *in vivo*, which is called Phytotherapy (Tiwari *et al.*, 2011).

2.6.2. Qualitative phytochemical analysis

The qualitative phytochemical analysis concern the phytochemical profiling of plant extracts using conventional standard processes for the determination of the existence of secondary plant metabolites, such as alkaloids, sugar, anthraquinones, flavonoids, terpenoids, saponins, tannins, and gums (Evans, 2015).

2.6.3. Chromatographic Techniques

Chromatography means separation of analytes in a mixture, based on the interaction with both phases, mobile and stationary. The division depends on differences in analytes' physical properties such as molecular size, load, and solvency. Based on mobile use, chromatography is dividing into gas and liquid. In the separation of natural products, chromatographical techniques were influential (Heinrich *et al.*, 2004, Cordell, 2014).

2.6.4. High-Performance Liquid Chromatography

HPLC (High-performance liquid chromatography) is a column chromatography commonly used in biochemistry and analysis in order to separate, recognize, and quantify active ingredient. HPLC primarily uses a column containing the filling material, a pump moving a portable stage through the column, and a detector showing the molecules ' retention times. Retention time depends on the relationships between the stationary phase, the analyzed molecules, and the used solvent (s). In combination with electronic library research and biologically active extracts, HPLC has become a powerful herbal medicine quality control method. It is now a chosen instrument to analyze most of the natural pharmaceutical products. There is one problem in the sense that both the machine and the consumable points of view are expensive (Gurib-Fakim, 2006, Malviya *et al.*, 2010, Mngeni, 2017).

2.7. Genetic diversity and DNA sequencing

2.7.1. Genetic diversity

Genetic diversity is the differences in the genetic make-up of different species and genetic variation in one species (Vellend, 2015). Individuals of a species share certain features by definition; however, genetic variation determines the specific features of people in the species. The most basic level of agro-biodiversity is genetic diversity, which is diversity within the species and populations (Newton *et al.*, 2010, Last, 2013).

2.7.2. DNA sequencing

The term DNA sequence refers to processes of determining the order of adenine, guanine, cytosine, and thymine in the nucleotide foundation in a molecule of DNA. At the beginning of 1970, academic scientists acquired the first DNA sequence by laboratory techniques based on two-dimensional chromatography (Munshi, 2012). DNA sequencing is becoming easier and

quicker by developing the dye-based sequencing process with automated analysis. A chemical procedure that breaks a finally marked DNA molecule partially at every repetition of the basis may be used to sequence DNA. The length and position of the labeled pieces are then defined. The effects of cleaving DNA on guanines, adenines, cytosine, and thymine are defined as responses, preferably, and cytosine is defined alone. If the product of these four reactions is solved by electrophoresis on a polyacrylamide gel, the DNA sequence can be seen from the pattern of the radioactive belts (Munshi, 2012).

Genotype sequencing should be able to differentiate between five groups for bi-allelic locus: (0:4), (1:3), (2:2), (3:1), and (4:0) (Last, 2013). Genotyping techniques such as direct Sanger amplification sequence have proven to be quantitative enough to allow discrimination on all copy numbers (Figure 11). Sequence variants in amplicons of target genes in the chromatogram repeat are recognized and then immediately quantified in the Sanger amplicon sequence (Koeyer *et al.*, 2010, Rickert *et al.*, 2002).

A nucleotide diversity index is called the rate at which nucleotide variations are detected between two homologous chromosomes that are randomly selected. The screening of additional chromosomes will recognize more polymorphisms, even while the Nucleotide Diversities Index stays continuous (Nasu *et al.*, 2002).

The DNA sample is re-sequenced using trace technique of the first, second, or third generation, and sequence variations are named by adding re-sequenced parts to a reference series. As found, variations in individual samples are defined by their reference place (genome) and are genotyped. The found versions and the outcomes of genotyping can be used for genetic analysis straight (Varshney *et al.*, 2009). More efforts are necessary for the design of unique primers and the optimization of the PCR amplification protocol for equal amplification of all alleles for a greater number of target genes. Thus, Sanger amplicon GBS has a limiting influence on speed and costs of variant discovery and genotyping for a large number of the genes (Uitdewilligen, 2012).

2.7.3. Genetic diversity of *Hyoscyamus niger*

The Hyoscyameae plant family consists of the *Archihyoscyamus, Anisodus, Atropa, Atropante, Hyoscyamus, Physochlaina, Przewalskia*, and *Scopolia* genera. Although Hyoscyameae's nonphysical support is strong, the taxa's relationships in the family are uncertain (Sanchez-Puerta and Abbona, 2014).

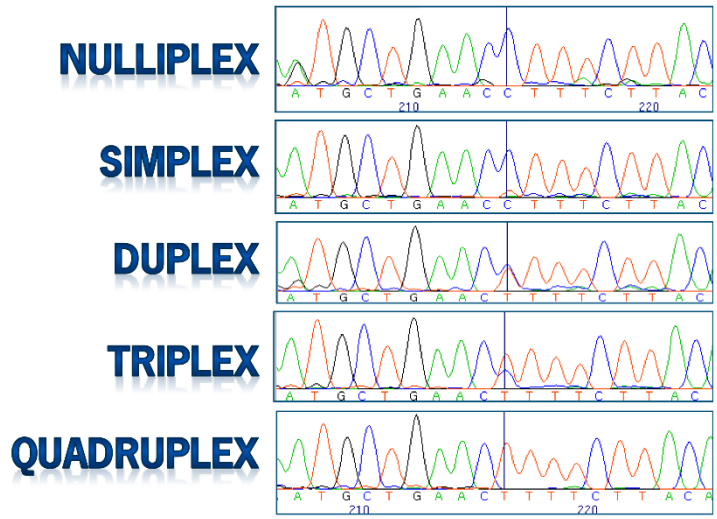

Figure 11: Genotype-sequencing example of a gene using Sanger amplicon sequencing (source: (Uitdewilligen, 2012))

The morphological isozymes were used in the genetic fingerprinting of *Hyoscyamus* species, total protein in the seed storage, and randomLy enhanced molecular markers of polymorphic DNA (RAPD) (Sheidai *et al.*, 2000, Sharifi *et al.*, 2006, Mahfouze and Ottai, 2011). But DNA-based markers are strong ways to distinguish differences in germplasm crops and to study developmental relations. Confident information on genetic identity and genotype relationship are needed for the development of core correlations for germplasm maintenance (Fatemeh Nejadhabibvash, 2012). The phylogeny of the Hyoscyameae family is elucidated by morphological characteristics, alkaloid-biosynthetic trajectories, cytological characteristics, and a few molecular markers (Knapp *et al.*, 1997, Tu *et al.*, 2005).

The next generation of sequencing has proven to be a quick and efficient way to sequence the *Hyoscyamus niger* organellar genome. *H. niger's* chloroplastic genome had a circular 155 720 bp molecule in length and a quadripartite structure comparable to the most terrestrial plant chloroplast genomes (Figure 12), (Tu *et al.*, 2010, Sanchez-Puerta and Abbona, 2014).

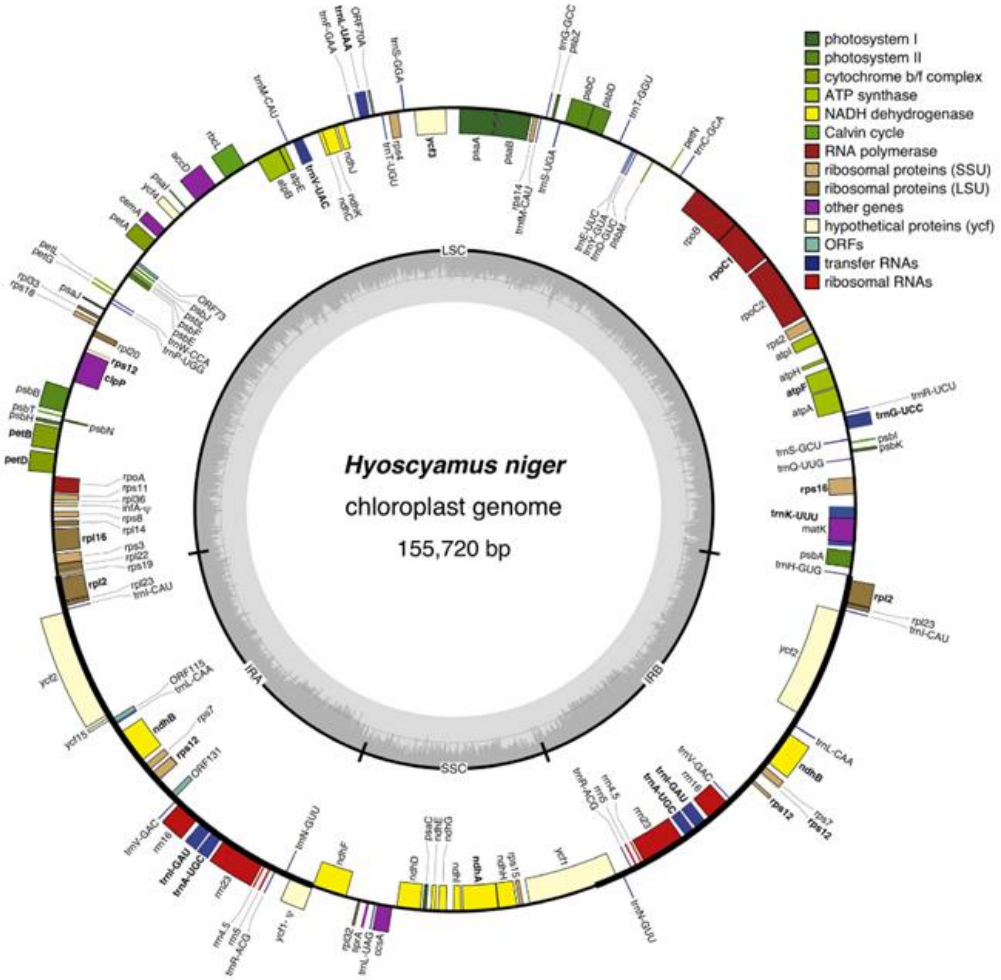

Figure 12: ***Hyoscyamus niger,*** **Chloroplast genome. The reversed replays are stated in large and small areas. The introns are made of courageous genes. In and out of the circle are transcribed genes, both clockwise and counterclockwise. Color marks are given to genes belonging to different functional groups (Source: (Sanchez-Puerta and Abbona, 2014)).**

2.7.4. Genetic diversity of *Sesamum indicum*

The genetic variability of Sesame has been studied by isozymes previously and numerous DNA markers, among them RAPD, ISSR, and AFLP (Isshiki and Umezaki, 1997). SRAP is a successful technique of genetic diversity characterization because it has a high degree of productiveness, discrimination, and a high polymorphic rate (Pham *et al.*, 2008, Kim *et al.*, 2002). SSR can readily be created at a reduced price, often have suspicious features, and are more useful for the assessment of comparative mapping, genetic diversity, and MAS breeding since it is derived from transcripts (Laurentin and Karlovsky, 2006, Zhang *et al.*, 2010).

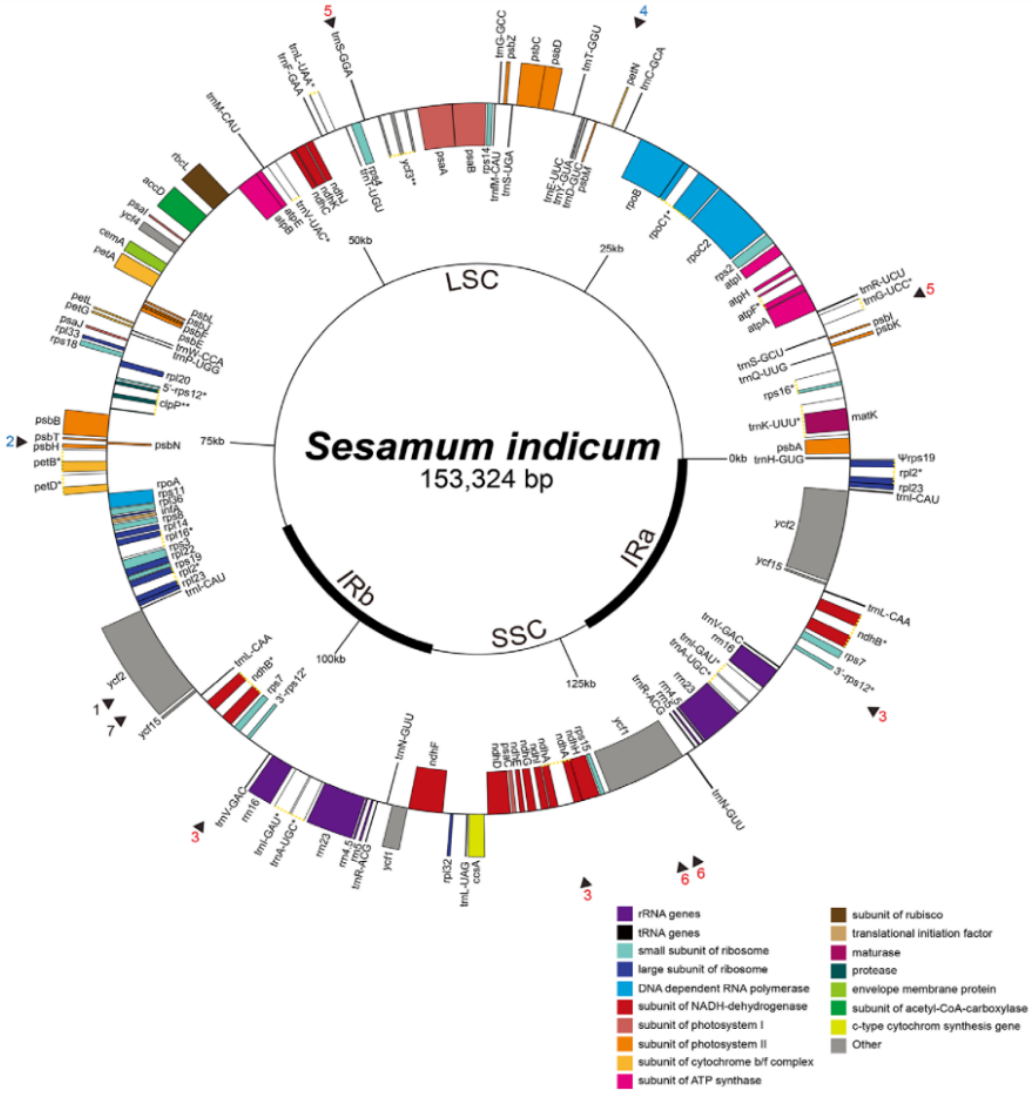

Figure 13: *Sesamum indicum* cp genome (The gene map). In the circle, genes are transcribed in the direction of the clock while those drawn outside the circle in the counterclockwise direction are transcribed. The asterisks are labeled for the intron-containing genes. The numbers at the end of the circle show seven repeat places, including immediate (black), palindrome (blue), and spread (red), respectively (Source: (Dong-Keun Yi, 2012)).

The entire *S. indicum* cp sequence is 153,324 bp in length, 58 percent coding areas, and 42 percent non-coding areas. A total of 114 genes, including eighty protein-coded gens, thirty RNA transfers, and four ribosomal RNA genes, are contained within the *S. indicum* chloroplast genome (Figure 13), (Dong-Keun Yi, 2012).

While *S. indicium* is mainly grown in developing countries, the majority of research focuses on sesame nutrients and foods. ISSR and EST tags for the development of genetic maps for sesame were most recently created. Until now, the sesame chloroplast (cp) genome sequence has not been investigated, and therefore the whole *S. indicum* cp genome sequences were produced and

described for their adequacy to be applied in future genetic engines as cp genome vector sequences (Wei *et al.*, 2011).

The chloroplast genomes contain wealthy genetic and phylogenetic data. Several latest studies have thus used cp genome data to build the phylogeny of angiosperm (Shinozaki *et al.*, 1998). cp genomes from multiple groups of crops and algae have been recorded with more than 170 species, including many plant species (Moore *et al.*, 2007, Linhai Wang, 2014).

CHAPTER THREE

3. MATERIALS AND METHODS

This study was conducted at Molecular Phytopathology and Mycotoxin Research Division, Georg-August-Universität Göttingen, during the years 2015-2017. The details of the experimental materials and methodology for the experiments are presented in this chapter.

3.1. Laboratory Instruments

These instruments have been used during the present study; Analytical Balances, Half-micro and analytical balance, pH-meter inoLab, pH-Electrode, Desiccator, Hotplate, and magnetic stirrer, Glassware, Drying Oven, Ultrasonicator, UV-Lamp, Rotary evaporator, Vacuum pump, Centrifuge, Micro Centrifuge, Freeze dryer, Vacuum pump, Speedvac, Cooling trap, Vacuum pump, Magnetic stirrer, Preparative HPLC, Analytical HPLC, HPLC-MS, NMR, and Lyophiliser.

3.2. Media and Buffer Composition:

3.2.1. Potassium phosphate buffer

It was prepared by mixing 136 g of KH_2PO_4 with 100 mL deionized water in a flask. The volume was finalized to 1000 mL. Then the pH was adjusted to 6.0, finally autoclave the mixture.

3.2.2. Nematode growth medium (NGM)

Nematode growth medium was prepared by mixing 3 g of NaCl, 20 g of agar, 2.5 g of peptone, and bring to 1 L with deionized H_2O (For liquid medium, exclude the agar). Autoclave for one hour, let cool for one hour in a 55°C water bath. Add 1 mL of 1 M $CaCl_2$, 1 mL of cholesterol (5 mg/mL in ethanol), 25 mL of 1 M KPO_4 (pH 6.0), and 1 mL of 1 M $MgSO_4$ mixing well after each addition complete the volume to 1000 mL (Stiernagle and Maintenance, 2006).

3.2.3. M9 buffer

M9 buffer was prepared by dissolving 6 g of Na_2HPO_4, 3 g of KH2PO_4, and 5 g of NaCl in 1 L of H_2O. Autoclave for 20 min. Add 1 mL of 1 m $MgSO_4 \cdot 7H_2O$. The volume was completed to

1000 mL. Stored at room temperature. After 2 weak, should be checked for contamination before uses (Stiernagle and Maintenance, 2006).

3.2.4. Egg Lysis solution:

For the preparation of 25 mL Egg Lysis solution, 5 mL of Fresh Clorox (6-14% bleach), has been mixed with 1.25 mL of 10 N NaOH and the volume was completed to 35 mL by adding 18.5 mL of sterile H_2O. This mixture must be prepared fresh before each use (Stiernagle and Maintenance, 2006).

3.2.5. LB medium

For preparing LB medium 10 grams tryptone, 5 grams yeast extract and 5 grams sodium chloride were dissolved in a one-litter conical flask, add 15 grams agar, and adjust the pH to 7.5 and the volume was completed to 1000 mL then autoclave before uses.

3.2.6. S-basal medium

S-basal medium was prepared by dissolving 5.9 g NaCl, 50 mL (1M) potassium phosphate, the volume was completed to 1000 mL using deionized water, then autoclave before uses.

3.2.7. YEB media:

YEB media was prepared by dissolving 1g/L yeast extract, 5 g/L Peptone, 5 g/L Beef extract, 5 g/L Sucrose, 4.5 g/L $MgSO_4$, 15 g/L (w/v) agar and adjusted to 7.2 pH. Finally, autoclaved and used after cooling or store it at 4°C until use.

3.2.8. APM (005)

APM (005) medium was prepared by dissolving 2,0 g/L $(NH4)_2SO4$, 5,0 g/L Bacto Yeast extract 5,0 g/L NaCl, 0,5 g/L Casamino acids, 8,0 g/L D-Mannitol, 15,5 g Agar, the volume was completed to 1000 mL using Milli-Q-water, Autoclave the medium let the medium, cool to 50°C and then add 0,1mg/L Biotin (pH6,6).

3.2.9. CTAB-buffer

For preparing CTAB-buffer, 7,4g Na-EDTA was dissolved in 50 mL 0,4 M stock solution, then 23 g Sorbitol, 10g N-Laurylsarcosine, 8g CTAB, 47 g Sodium Chloride, 10 g Polyvinylpyrrolidone (25.000 - 40.000), 10 mL (1 M) Tris stock solution, in 800mL was

dissolved in distilled water and adjust the with NaOH pH to 8.0, fill up to 1000 mL distilled water then add 20 mg/mI Proteinase K in (TE buffer) and 10 mM Tris. 1 mM EDTA, pH 8.0 TE buffer then Mercaptoethanol were mixed.

3.2.10. One Tag PCR (High fidelity)

Prepared by mixing 5 µL, 5xOne Tag Standard Reaction, 2 µL (2.5 mM) dNTPs, 0,5 µL 10 µM Forward Primers, 0,5 µL 10 µM Reverse Primers, 0,13 µL One Tag Hot Start DNA polymerase, 10 µL Template DNA, 6,88 µL Bidest Water, fill it to total valium 25 µL.

3.3. General Solutions

The following solutions were used in this study:

3.3.1. Ethidium Bromide (EtBr)

Ethidium Bromide was prepared by dissolving 0.1 g of ethidium bromide powder in 10 mL of distilled water. The container was wrapped in aluminum foil for protection of the solution from sunlight and stored at 4°C safely before use.

3.3.2. 70% Ethanol

70% Ethanol was prepared by dissolving 70 mL of pure ethanol in 30 mL of distilled water.

3.3.3. 10% Ammonium Persulfate (APS)

10% Ammonium Persulfate was prepared by dissolving 1 g of ammonium persulfate in 10 mL of distilled water, separated into 2.5 mL aliquots, and stored at 4°C until required.

3.3.4. Loading Dye

Loading Dye was prepared by dissolving 0.025 gr bromophenol blue and four grams sucrose in 10 mL water.

3.3.5. 20% SDS

20% SDS was prepared by dissolving 20 gm of sodium dodecyl sulfate in 100 mL of water.

3.3.6. 10X Running Buffer

10X Running Buffer was prepared by dissolving 303 g Tris, 1.44 Kg glycine and 100 g SDS into distilled water to a final volume of 10 L. Dilute to 1X concentrate in distilled water before use.

3.4. Plant Material and Seed collection

The seeds of seven varieties from *Hyoscyamus niger* and twenty-five varieties of *Sesamum indicum* were investigated during this study (Figures 14 and 17). The seeds of *S. indicum* were collected from 25 distinct regions in which 16 varieties were provided by Karlovsky lab-Göttingen University, and 9 varieties were collected from Iraq, and *Hyoscyamus niger* seeds were collected from 7 distinct regions, from which one variety was provided by Göttingen botanical garden. Two varieties were commercial products provided by Iran. Four varieties were collected from Kurdistan mountains in Hawraman and Penjwen areas (Figures 16 and 17) and (Tables 1 and 2), the identity of *Hyoscyamus niger* plant was confirmed by Assistant Prof. Dr. Saman A. Ahmad (Department of Crop Production, College of Agricultural Engineering Sciences at the University of Sulaimani).

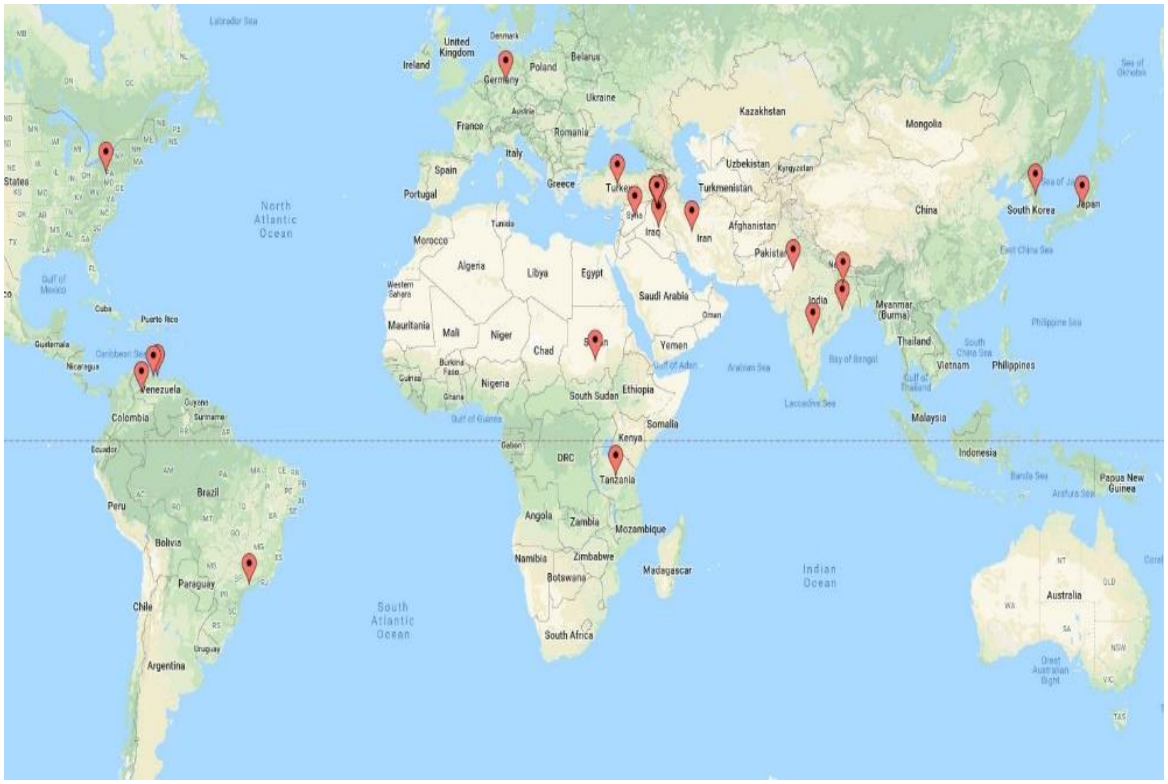

Figure 14: *Sesamum indicum* varieties distribution (easymapmaker)

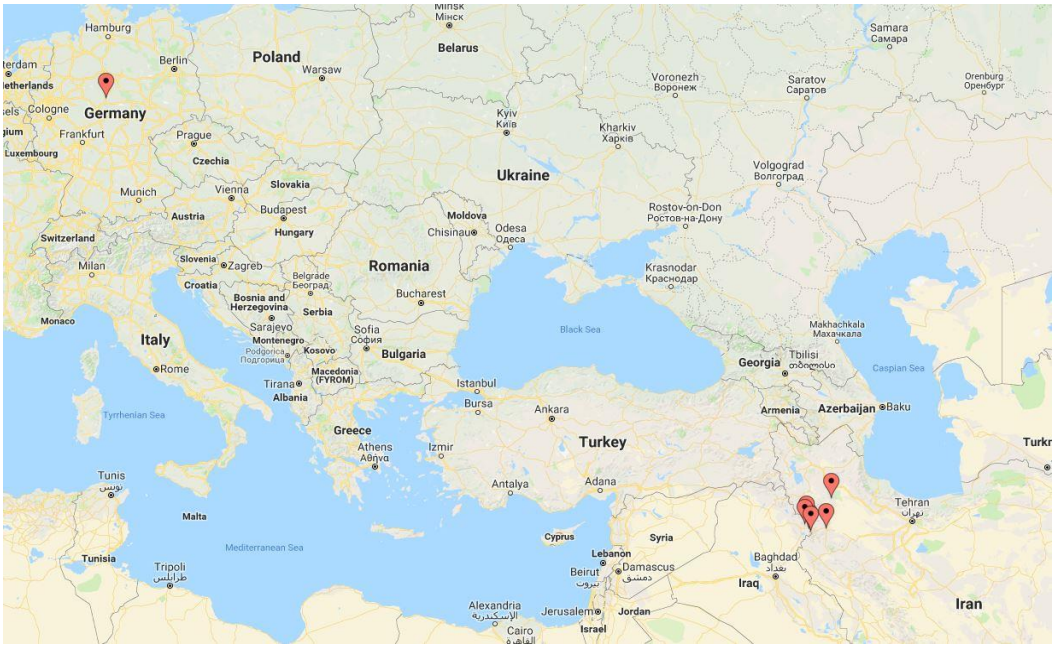

Figure 15: *Hyoscyamus niger* varieties distribution (easymapmaker)

3.4.1. Seed germination

One of the major problems of *Hyoscyamus niger* seed germination is the hard dormancy breakdown. The seeds of *Hyoscyamus niger* and *Sesamum indicum* were soaked in different concentrations of gibberellic acid (GA3) solution (0.0, 2.5, 25, and 250 mg/L) for 12h. At room temperature (25±0.5°C) for dormancy breaking and accelerating the germination. The experiments were set up in a completely randomized design. For each treatment, three Petri dishes were used (each Petri dish representing a replicate), and there were ten seeds replicate.

Figure 16: *Hyoscyamus niger* L., at flowering stage grown under specific laboratory condition (Source: by the Author)

Seeds were superficially sterilized, by washing with distilled water and immersed in 70% ethanol for thirty second followed by 6% commercial bleach (5% sodium hypochlorite) for 15 min then washed three times (5 min) with sterile deionized water prior to placing onto half-strength (½ MS) Murashige and Skoog medium and or germinated on a cotton pad or sterilized filter paper under controlled conditions (25°C with a 16/8 h photoperiod at 4000 Lux light intensity) in the disposable Petri dish (Bekheet, 2013).

Figure 17: *Sesamum indicum*, at flowering stage grown under specific laboratory conditions (Source: by the Author)

All the media were autoclaved at 122kPa within 120°C for 20 min. After cooling down the media to 37°C 25 mL, the media was poured into the 9 cm Petri dishes. Laminar flow conditions were used for all experimental protocols. The Petri dishes were sealed with laboratory Parafilm and transferred to the growth room at 23±2°C in the dark, to evoke germination for 3 days then the seeds were cultured under the light/dark condition with 16/8-h cool-white fluorescent light. After 24 h, the number of germinated seeds was counted, and then, the seeds were observed every two days for germination for a period of 30 days.

Table 1: Varieties of *Hyoscyamus niger*, used during this study

Varieties Name	Source	Origin
KRI Hawraman	Collected by ZJK	Tawella/Iraq
KRI Hasanawa	Collected by ZJK	Hasanawa/Iraq
KRI Pinjwen	Collected by ZJK	Pinjwen/Iraq
KRI Daray Mar	Collected by ZJK	Daray Mar/Iraq
Iran Takhte	Frome Seed company*	Takhte/Iran
Iran Isfahan	Frome Seed company*	Isfahan/Iran
Germany 1	Göttingen botanical garden	Göttingen/Germany

*Pakan Bazr Isfahan Company Supplier

Table 2: Varieties of *Sesamum indicum,* used during this study

Varieties Name	Source	Origin
India 1	Karlovsky Lab*	Rajasthan/India
India 3	Karlovsky Lab*	Bihar/India
India 7	Karlovsky Lab*	Odisha/India
India 8	Karlovsky Lab*	Hyderabad/India
Korea 1	Karlovsky Lab*	Korea
Turkey	Karlovsky Lab*	Turkey
Syria	Karlovsky Lab*	Syria
Sudan 2	Karlovsky Lab*	Sudan
Africa 3	Karlovsky Lab*	Tanzania
Japan 2	Karlovsky Lab*	Japan
China 2	Karlovsky Lab*	China
Vzla 51	Karlovsky Lab*	Guacara/Venezuela
UCV-1	Karlovsky Lab*	Caracas/Venezuela
Maporal	Karlovsky Lab*	Maporal/Venezuela
Inamar	Karlovsky Lab*	Inamar
UCLA 1	Karlovsky Lab*	Altoona
Sumer S1a	Agriculture Directory Erbil**	Erbil/Iraq
Sumer S1b	Agriculture Directory Erbil**	Erbil/Iraq
Sumer S2a	Agriculture Directory Erbil**	Erbil/Iraq
Sumer S2b	Agriculture Directory Erbil**	Erbil/Iraq
KRI local Hawler 1	Agriculture Directory Erbil**	Erbil/Iraq
KRI local Ranya 1	Agriculture Directory Erbil**	Rania/Iraq
KRI local Hawler 2	Agriculture Directory Erbil**	Erbil/Iraq
KRI local Ranya 2	Agriculture Directory Erbil**	Rania/Iraq
Iran 1	From Seed company***	Isfahan/Iran

* Germplasm Bank of Centro Nacional de Investigaciones Agropecuarias (CENIAP), Venezuela

** Ministry of Agriculture, Regional Government of Iraqi Kurdistan, General Directorate of Research, Extension, and Training

*** Pakkan-bazr Company, Iran, Isfahan

3.4.1.1. Aseptic seed germination

The seeds of *H. niger* and *S. indicum* after surface sterilization were cultured on MS medium containing vitamins, free from plant growth regulators, and incubated inside the growth chamber under controlled condition (25°C with a 16/8 h photoperiod at 4000 fluorescent light intensity) in the experimental glass tube. After germination and formation of seedlings with true leaves, individual and healthy, uniform plants were prepared for hairy root culture.

3.4.1.2. Non-Aseptic seed germination

Hyoscyamus niger and *S. indicum* seeds were surface sterilized and germinated on a cotton pad or sterilized filter paper under controlled conditions (25°C with a 16/8 h photoperiod at 4000 Lux light intensity) in the disposable Petri dish. After seed germination, the seedlings with three true leaves individually, transplanted into experimental pots 30 cm diameter. The soil composition was; 50% sand, 25% silt, 23% clay, and 2% compost. The pots kept in the growth room, and after 50 days, the flowering started between in the varieties of both plant species.

3.5. *Caenorhabditis elegans*

The N2 wild-type *Caenorhabditis elegans* nematode and OP50 *Escherichia coli* strains were kindly provided by the (Faculty of Forest Sciences and Forest Ecology) in February 2015 by Dr. Hujia at the University of Göttingen. The *C. elegans* were cultured on nematode growth medium (NGM) with OP50 *Escherichia coli* under the standard condition in Petri dishes (9 cm in diameter) and maintained in an incubator at a constant temperature of 20°C (Brenner, 1974).

3.5.1. Basic methods

The preparation of all the media and buffers regarding anthelminthic activity was done according to the book *Caenorhabditis elegans*: a modern biological analysis of an organism (Henry F. Epstein, 1994), and The Genetics of *Caenorhabditis elegans* (Brenner, 1974).

3.5.1.1. Bacterial Preparation

Lysogeny broth (LB) medium was prepared and inoculated with *E. Coli* strain OP50; the suspension was cultivated overnight on the shaker incubator at 37°C and transferred to sterile centrifugation tubes (15 mL) and stored in the fridge.

3.5.1.2. Preparing NGM plates

NGM medium was prepared by mixing the gradients mentioned previously in section (3.2.2.), then pipetted to a Petri dishes with a volume of 5-6 mL for 60 mm. After medium solidification, 30 µL of bacterial suspension in the LB medium was added for 60 mm PD and spread with sterile spreader plates. The plates were inoculated overnight at 37◦C for two days at room temperature and stored in the fridge (Brenner, 1974).

3.5.1.3. Transfer of Nematodes

3.5.1.3.1. Chunking

Nematodes were transferred to a fresh medium in 2-3 days interval to maintain viability, parallel to the time for the lack of food and plate overcrowding. Using worms from too old plates is not recommended while it can be used.

Transferring (Chunking) worms to a new plate by a sterilized spatula, 1 to 2 cm piece of the old worm-covered agar removed and placed upside-down on the fresh bacteria-covered plate. The new cultured plate could be used for 1-7 days after chunking, based on the size of the chunked piece, transferring the higher number of worms, the faster they would reproduce and cover the new plate. And the temperature of the incubator because the worms grow faster at higher temperatures. To avoid contamination of the fresh agar plate, the chunked transfer should be performed as fast as possible (Matilda Backholm, 2015).

3.5.1.3.2. Picking up

The nematodes were picked up from the surface of an appropriate agar plate, into a drop of buffer. This should be done carefully with an instrument that can be created by melting the tip of a Pasteur pipette in the flame burner and placing a platinum wire ∼2-3 cm long, ∼2 mm thick, into the end of the glass. The pipette then sticks to the wire and hold it tightly. The picking up process is done under a dissecting stereomicroscope by placing the sharp picker carefully under the worm of interest, and then scooped up the nematodes to put it in the drop of buffer. The picking techniques should be done carefully since worms were easily injured and killed, as the worms die within 10s of seconds after being placed on a dry surface (Matilda Backholm, 2015).

3.5.1.3.3. Liquid culture transfer

The appropriate nematode plate was washed-up with M9 buffer, under aseptic conditions inside a laminar airflow, and placed in a 15mL falcon tube; then, the *C. elegans* nematodes were

washed up three-time centrifuging interval. Finally, the precipitated nematode placed on a new NGM plate with OP50 *E. coli*.

3.5.1.4. Decontamination

The NGM agar is designed specifically to provide an excellent growth environment for *E. coli* bacteria, and this will make an appropriate condition for contamination with bacterial, fungal, and yeast growths. Small contaminations can be eliminated by chunking worms found away from the growth and then sterilely transferring them to new plates. Meanwhile, aggressive bacterial, fungal, and yeast contaminants require a different approach via treatment with specific antibiotics (Coburn, 2012).

3.5.1.5. Baermann Funnel Nematodes Isolation

This is the classical method to isolate and recover *C. elegans* nematodes, and it is useful to separate nematodes deprived of contamination by *E. coli*. A rubber tube fitted onto the stem of a funnel and closed the tube with a clamp and fill the rubber tubing with M9 buffer solution up to the neck of the funnel.

Line the funnel shaft with a filter tissue, fill with the cultured nematodes agar. Fill the funnel with M9 solution or water until the sample is submerged. Active nematodes crawl through the filter tissue and then precipitate at the end of the tubing. Collect them by opening the clamp after a few hours (Figure 18).

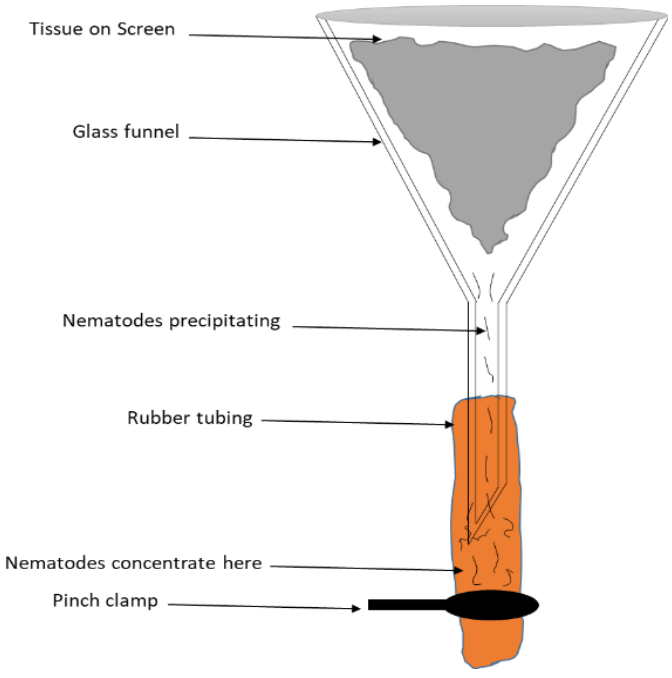

Figure 18: Baermann funnel method. The *C. elegans* nematodes sway towards the base of the funnel. This may take a few hours (Source: photograph by the Author).

3.5.1.6. *Caenorhabditis elegans* larvae

3.5.1.6.1. Synchronization

Caenorhabditis elegans nematodes were grown on ten to fifteen standard NGM plates with OP50 *E. coli* bacteria for about three days until enough gravid Adults were present. The Adult nematodes and eggs were washed off in M9 buffer and transferred to a 50mL falcon tube allowed to chill on ice for 15-30 min. The Falcon tube was centrifuged at 2500 rpm, 4^{o}C for 3 min. The supernatant was removed using 10 mL with a 2 mL pipette without disturbing the pellet. 10 mL of bleach solution (625 µL 4M NaOH, 1500 µL concentrated bleach and 7875µL distilled water) added to the worm pellet and the tube agitated. After approximately 3 min, and every minute after that, a 10 µL sample was removed and examined under a dissecting microscope. Once the adult worms began to lyse completely and the eggs been released, the falcon tube was filled to the top with ice-cold M9 buffer. The tube then immediately centrifuged at 2000 rpm, 4^{o}C for 2 min. The pellet of eggs was washed for three further times.

Finally, the supernatant was totally removed, and the pellet re-suspended in approximately 5-7 mL of S-buffer and transferred to a 5 cm diameter petri dish. The eggs were incubated overnight at 20^{o}C to allow the eggs to hatch. The following day 10 µL of the L1 suspension was removed, and the number in L1 larvae counted. The experiment repeated 3 times, and the mean number of worms per 10 µL was calculated (Laing, 2010).

3.5.1.6.2. Nematodes flotation

C. elegans nematodes have a specific gravity and can be separated by precipitation in a solution with a specific gravity (Hooper, 1986). This technique can be used to separate the *C. elegans* nematodes according to their size, in which the adult and the largest size precipitate faster than the smallest size L1 larva that moves and swims more quickly.

The newly grown nematode plate was washed out using M9 buffer or sterilized tap water, and the nematode is washed three times, then the nematode solution was placed in Burette Glass 50 mL Grade. The nematode solution was collected every 5 minutes and examined under the dissecting microscope to determine their stage. This method is a powerful method for the quick separation of *C. elegans* nematodes according to their life stage.

3.6. Hairy Root Induction and Culture Conditions

Leaves from *in vitro* grown pre-established plant cultures of *Hyoscyamus niger* L., and *Sesamum indicum* L., were maintained on solid hormone-free MS medium and used as explants for induction of hairy roots as described in section (3.4.1.1) (Murashige and Skoog, 1962).

3.6.1. *Agrobacterium rhizogenes* strains and maintenance

Agrobacterium rhizogen A4 was kindly provided by Dr. Kirsi-Marja Oksman, VTT Industrial biotechnology, and Food Solutions, Finland. And *A. rhizogenes* ATCC 15834 was kindly provided by Prof. Dr. Jutta Ludwig-Mueller, Technical University Dresden, Germany, was used for hairy root induction.

Agrobacterium rhizogenes ATCC 15834 was grown in Yeast Extract Beef (YEB) medium (Hooykaas, 1977), (0.1% yeast extract, 0.5% peptone, 0.5% meat extract, 0.5% sucrose, 0.049% $MgSO_4*7H_2O$, 1.5% Agar, pH 6.8-7.2), at 25-26℃ for 36 h in the dark on a shaker incubator (100 rpm) and *Agrobacterium rhizogenes* A4 was grown in (APM 005) (2.0g $(NH_4)_2SO_4$, 5.0 g Bacto Yeast extract, 5.0 g NaCl, 0.5 g Casamino acids, 8.0 g D-Mannitol, 0.1mg/L Biotin, 15.5 g Agar), 1000 mL Milli-Q-water, pH 6,6 at 28°C for 36 hours in the dark on a rotatory shaker (100 rpm). A single colony in 5 mL (YMB) or (APM 005) media was used for the preparation of a freshly bacterial suspension. then incubated in the dark for 36 hours on an orbital rotary shaker with 200 rpm at (25-26°C) or (28 ± 2°C) before any experimentation. A spectrophotometer was used to determine bacterial growth on the suspension by its optical intensity at 600 nm. Usually, bacterial suspension culture having 1.0-1.3 OD600 is used for hairy root induction and collected by centrifugation at 5000x g for 15 min, then re-suspended in MS medium (Murashige and Skoog, 1962), and set to the OD600 of 1.3.

3.6.2. Hairy Root initiation

3.6.2.1. Co-Cultivation of bacteria along with wounded plant tissue

The explant leaves were collected from four-week-old plants grown from superficially sterilized seeds, as described in section (4.3.1.1) under aseptic conditions in a laminar airflow cabinet (Bekheet, 2013). The harvested, wounded leaf explants were immersed in sterile distilled water instead of bacterial suspension and incubated in the same conditions were served as a negative control (Ooi *et al.*, 2013). Leaf explants grown on 1/2 MS solidified medium with 0.7% agar were cut into pieces of (0.5X0.5) cm and co-cultivated by dipping in 24 hours old grown bacterial suspension with (OD600 1.0 -1.3) diluted in 1/2 MS medium for five min. For control, similar experiments cared out using distilled water instead of bacterial culture. The leaf segments were dried with sterile filter paper to remove the exceeded bacterial colony and placed onto semisolid phytohormone-free MS medium with 0.7% agar supplemented with 2.5 % (w/v) sucrose and incubated in the dark at 22°C (Vikas, 2013).

3.6.2.2. Transfer of plant tissue to antibiotic-containing medium

After 48-72 h of incubation at 25 ± 2ºC in the dark, the co-cultivated and control explants were transferred to hormone-free solid MS medium containing Cefotaxime antibiotic at a concentration of 500 mg/L to remove the bacterial growth. Antibiotic treatment was repeated 3-4 times until the explants were free of bacteria, and the bacterial growth on the medium surface was not visible (Vikas, 2013).

3.6.2.3. Determination of transformation frequency

The hairy root culture cultures were observed periodically from 10-60 days to record the transformation frequency, which is recorded after 60 days. The frequency of transformation can be determined by counting the number of lives injured that show the appearance of hairy roots in a total number of explanatory wounds. The percentage of transformation frequency was recorded as follows (Vikas, 2013):

$$\textit{Transformation frequency} = \left(\frac{\text{Number of explants Showing Hairy Root}}{\text{Total Number of explants}} \right) \text{X100}$$

3.6.2.4. Maintenance of Hairy Roots

The newly appeared hairy roots were removed individually and transferred to both liquid and solid hormone-free MS medium containing cefotaxime antibiotics at the concentration of 250 mg/L for the complete removal of the *Agrobacterium rhizogenes* bacterium. These excised roots were frequently sub-cultured at four weeks interval in a fresh medium with a gradual lowering of the antibiotic concentration (Vikas, 2013).

3.6.3. Fexed Air Shaking Flask culture

For the suspension culture, 100 mg FW roots were weighed inside a laminar airflow and transferred to 300 mL conical flasks filled with 50 mL MS medium supplemented with 2.5% sucrose and 250 mg/L cefotaxime. Root cultures were grown on a shaker at 100 rpm in the dark at 25 ± 2°C and subcultured every 3 weeks. After the third subculture, 6 well-grown hairy root clones were selected for further analysis.

3.6.4. Novel Small Bubble Bioreactor

The body of the reactor (Figure 19a, part 3) was made of a glass cylinder with an external diameter of 90 mm, height 270 mm, and wall thickness 2.5 mm. The external draft tube of 18

mm diameter and 75 mm height was connected with the reactor body at two positions via glass tubes, as shown in (Figure 19a and b). The bottom connector accommodated a glass tube with a frit serving to supply air in the airlift operation mode (Figure 19a part 2). The top connector was used for air supply in bubble aeration mode. In the assembled reactor, a clearance of 20 mm width in the support cylinder (Figure 19 apart 1) faced the draft tube (Figure 19b) to allow the exchange of growth medium between the body of the reactor and the draft tube and facilitate air supply in the bubble aeration mode. The lower part of the reactor was inserted into a cover made of opaque plastic to shield the roots from light (Figures 19a part 5). For visual inspection of the roots, the cover was removed (Figure 19a part 3). The reactor was covered with a glass lid (Figure 19a part 6), which was placed loosely over the body or the reactor (Figure 19a and c) or sealed in place with Parafilm (Figure 19c). Petroleum jelly was occasionally used instead of Parafilm. A glass Petri dish of internal diameter 91-92 mm proved suitable as a lid. The volume of growth medium was 450 mL. The airflow rate in the bubble mode was 670 mL/min. The bubble bioreactors, which were sterilized by steam (120 ^{o}C, 0.1 Mpa) before use equipped with (80 X 70 mm) glass basket and (4 mm mesh) for root immobilization. The growth vessel was filled with (450 mL working volume) medium, inoculated with 1000 mg FW of 1cm long hairy roots, and the culture maintained for 30 days in Bb. The biomass and medium samples from the bioreactor collected after unloading the installation on the respective day of the experiment. The Hairy root plotted dry on sterilized filter paper, then lyophilized for three days, then kept at (-20°C).

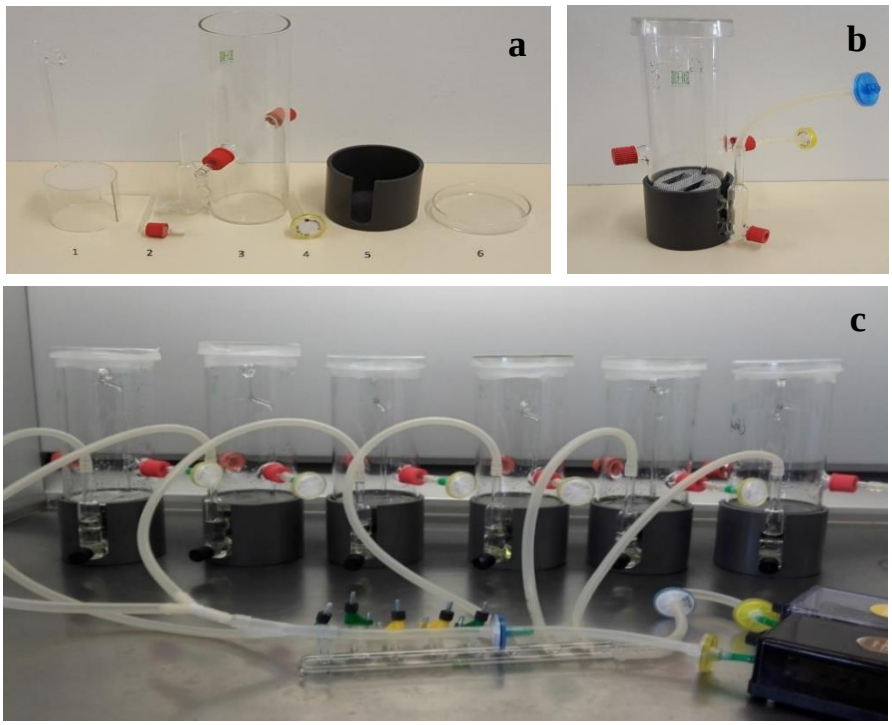

Figure 19: Bubble Bioreactor; a. separate part of the bioreactor (1-glass stand and plastic stage, 2-adapter, 3-reactor body, 4-air filter, 5-base stand, 6-had cover). b. Single reactor. c. Complete Bioreactors device.

3.6.5. Operation of the reactor

The suitability of the reactor for the operation was tested in bubble operating mode, the bottom connector was locked with a screw cap, and the upper connector was attached to the source of sterile air, which was forced into the reactor via the upper connector. When the reactor was loosely covered with a glass dish, air streaming out beneath the lid prevented contamination. A port equipped with a sterile filter was used as air exhaust when the lid was tightly sealed. As a low-cost air source, we used aquarium pumps connected to the reactors via disposable syringe filters with a pore size of 2 µm. The same filter was attached to the exhaust port of the reactor in (Figures 19B and C). Reactors covered with inverted Petri dishes without a seal seldom became contaminated. The air-lift mode was used for growing intact plants in sterile hydroponics. Hairy root cultures are known to exhibit inhomogeneous growth and inferior yields when growing in air-lift reactors; therefore, and bubble aeration mode was used (Caspeta *et al.*, 2005).

3.6.6. Harvest of hairy roots and hairy root exudates from the bioreactor

Hairy root cultures were incubated under continuous aeration for 30 days. Roots were separated from medium, blotted dry with sterilized filter paper, and their fresh weight was determined. The samples were kept at -20°C overnight, lyophilized for 72 hours, and their dry weight was determined. Medium separated from hairy roots was cleared by centrifugation for 10 min at 4,000 rpm to remove root cells and debris and kept at -20°C.

3.7. Biomedical Activity

3.7.1. Collection of Plant material

Collection of seed, root, and hairy root samples of *H. niger* and *S. indicum*, from both cultivated and wild-growing medicinal plants, were done as described in section (3.4). Root samples were collected from mature plants grown from sterilized as described in section (3.4.1.2). The roots were separated from the mature plant and washed gently with tap water to remove the clay and cleaned completely then blotted dry with lean filter paper. The hairy roots from all plant varieties were initiated as described in section (3.6), followed that the HR was separated from MS medium and plotted dry and weighted then Stord directly at -20^{o}C. All plant parts were stored at -20^{o}C for 24 hours and dried by using a freeze dryer (Lyophilizer) for 72 hours. After freeze-drying, the samples were stored under -20^{o}C until uses.

3.7.2. Preparation of Plant Extracts

3.7.2.1. Methanolic Extract

A modified method was applied for the plant's extraction (Gorkem Dulger and Dulger, 2015). The seeds of the plant were extracted using an aqueous 60% methanol/water. 10 g of the Seed, Root, and Hairy Root materials were extracted in flasks placed in an ultrasonic bath first with 50 mL solvent for 60 min, took with 30 mL solvent for 45 min, and finally with 20 more mL solvent for 15 min, the overall extraction taking 120 min. The three extracts were combined and filed to a final volume of 100 mL with an aqueous 60% methanol. The extract filtered, and the solvent was removed using a rotary vacuum evaporator with a water bath at the temperature of 40^{o}C. The collected, dried extract was stored in labeled sterile tubes at -20°C until it used.
Lethality assays were performed using *C. elegans*, nematode growth media NGM In 24-well plate (Parreiraa *et al.*, 2010), plant extracts stock solutions were made in DMSO. On the day of the experiment, the appropriate serial concentration of the extracts was prepared to contain between 90 and 100 synchronized (L1) larvae. Final concentrations of the compounds were added in the range of 0.00, 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500 µg/mL, and the maximum DMSO concentrations amounted to 3%. As a negative control, nematodes were exposed to the same conditions containing 3% DMSO. The nematodes incubated at 25°C for 48 h. Data were calculated from 3 independent experiments. The LC_{90} values are representing 90% lethality and LC_{50} values, representing 50% lethality, which calculated using (GraphPad Prism 7.0). For examination of the nematodes, a stereo microscope Stemi 2000-C (ZEISS, Germany), with magnifications of 13- to 100-fold, was used.

3.7.2.2. Extraction of Fixed Oil

The extraction of fixed oil was conducted with a modified Soxhlet apparatus. 100 g of powdered seeds were extracted for 5 h, and the solvent was evaporated by using a rotary evaporator at 30°C. The pure oil was collected in a small dark glass vial, maintained at -20°C until analyzed for fatty acid composition (Samira Alipour-Gougeh and Asgarpanah, 2015).

3.7.2.3. Extraction of Essential Oil

Essential oils (EOs) were Extracted using the modified hydro-distillation method by the Clevenger type apparatus (Figure 20), connected to a 1L round-bottom according to the

European Pharmacopoeia. The extraction process was conducted for three hours, while the solution was kept boiling. Hydro-distillation was run at a distillation rate of 3 mL/min, by using 25 g dry weigh seed to 150 mL distilled water. EOs were isolated using a Pasteur pipette and stored in the dark glass bottle at -20ºC until analysis (Millezi *et al.*, 2012).

Figure 20: Clevenger type apparatus (Source: by the Author).

3.7.3. *Caenorhabditis elegans* Nematode Collection and Preparing

Caenorhabditis elegans N2 wild-type strain and *Escherichia coli* (strain OP50), were kindly provided by Dr. Hujia, (Faculty of Forest Sciences and Forest Ecology, University of Göttingen), which maintained on Nematode growth plates with *E. coli* OP50 strain as a nutrition source on NGM medium. The poured Petri plates of NGM medium were seeded with 0.1-0.2 mL of overnight cultures of *E. coli* grown in Lauryl broth and allowed to grow into a lawn (Sant'Anna *et al.*, 2017). Chunking of different developmental stages nematodes (i.e., larvae, adult males, and hermaphrodites) along with few eggs were placed on the new fresh medium. Bleaching the adult's Nematodes according to the documented process after three days and seeding synchronized (L1) larvae stage onto OP50 seeded fresh ENG plates 48 hours before setting up an essay.

3.7.4. Bioassays

3.7.4.1. Direct Contact Bioassays (*Crude extract*)

Direct contact bioassays were conducted from newly extracted plant specimens (Seed, Root, and Hairy Root). The *C. elegans* L1 larva was resuspended in M9 and approximately 100 larvae in 100 µL of suspension in which 95 µL added to each well in a 96 wells microplate. The stock solution of the studied plant's crude extracts were prepared by dissolving 5 mg of the dry black sticky substances in 1mL of DMSO to get the initial concentration of 5 mg/mL, then by two-fold serial dilutions final concentration's of 1.95, 3.90, 7.80, 15.60, 31.25, 62.5, 125, 250, and 500 µg/mL of stock solution were prepared Five µL of the stock solutions were added to 95 µL of nematode suspensions in the M9 buffer. three percent of DMSO was used as a negative control, as previously described by Moshi and Mbwambo (Moshi and Mbwambo, 2005).
All bioassays analyses were performed in 96-well microtiter plates, covered with plastic film, to reduce volatilization, aluminum foil for providing total darkness, and maintained at 25±1ºC in BOD incubator, for 24 h. Dead and live nematodes were counted using an optical microscope (40x). Nematodes were considered dead after losing immobility even when physically stimulated. These assays were performed for nine plant varieties with three replicates using three plant parts (Seed, Root, and Hairy Root).

3.7.4.2. Essential oil and Fixed oil from *Hyoscyamus niger*

Essential and fixed oil, in *Hyoscyamus niger,* were assayed for anti nematocidal activity. *Caenorhabditis elegans* L1 & L2 Larve were resuspended in M9, and approximately 100 larvae in 100 µL of suspension were prepared. The extracts stock solutions were prepared in methanol, To obtain a final concentration 5µL of these solutions were added to 95 µL of nematode suspensions with 100 L1 at 1.95, 3.90, 7.80, 15.60, 31.25, 62.5, 125, 250, and 500 µg/mL. Methanol was used due to its high polarity and high solvent capacity. Control were performed with methanol 3% (methanol/nematode suspension v/v). To check for methanol-induced mortality, sterilized deionized water was used as a control. All bioassays were performed in microtiter 96-well plates, covered with plastic film, to decrease volatilization, and aluminum foil to provide darkness. The plates were maintained at 25±1ºC in the BOD incubator for 24 h. Live and dead nematodes were counted using an optical microscope (40x). Nematodes were considered dead when they lose mobility even when physically stimulated.

3.7.4.3. Thermal Release Bioassays

3.7.4.3.1. Plant material

All the experimental plants used in this experiment were listed in (Table 3).

Table 3: Plant varieties used in Thermal Release Bioassays

Varieties	Country of Source	Variety	Year of Collection
Hyoscyamus niger	Iraq	Hawraman	2015
Hyoscyamus niger	Iraq	Hasanawa	2015
Hyoscyamus niger	Iraq	Pinjwen	2015
Hyoscyamus niger	Iraq	Daray Mar	2015
Hyoscyamus niger	Iran	Takhte	2015
Hyoscyamus niger	Iran	Isfahan	2015
Hyoscyamus niger	Germany	Göttingen 1	2016
Sesamum indicum	India	India 7	2016
Sesamum indicum	Inamar	Inamar	2016
Sesamum indicum	Erbil	Sumer S2a	2016
Cicer arietinum	Iraq	*Cicer arietinum*	2016

3.7.4.3.2. Preparation of plant material

Plant seeds were freeze-dried by lyophilization for 72 hours and homogenized with Mixer Mills-MM400, weighed. Ten grams of the powdered material was prepared to be used in "Thermal Releaser".

3.7.4.3.3. Thermal Releaser

The body of the Device (Figure 21a) made of a glass desiccator with an external diameter of 200 mm, height 150 mm. There is a lead one internal flow tube of 5 mm diameter and 180 mm height, and one external draft tube of 5 mm diameter and 70 mm height within the body of the device was connected with the thermal generator, which is a conical flask of 1000 mL capacity (Figure 21b).

The conical flask was placed on a water bath, the test part of the device (Figure 21a) consisted of two layers, the lower part occupied with ice, and the upper one is a stage for holding the processed plate. Both parts of the device were connected via the thermal resist tube of 5 mm diameter supported with a control air pump to circulate the air (Figure 21). The temperature of both parts of the device was extra controlled by an electronic thermal detector (Figure 21c).

3.7.4.3.4. Working principle

Ten grams of the dried powdered seed were placed in the thermal generator (Figure 21b) of the device and flask to a specific tested temperature and a flask number of tested *Caenorhabditis elegans* placed on 15mL Petri dish and placed in the tested area without Petri dish lead. The air pump turned on that led to the air circulation between both device parts, the air comes from the bottom of the (part b) to the (part a) and returns to (part b) to be exposed to the tested nematodes. Barriers flask at the end of the upper tube to prevent direct exposer of the air to the nematode. For 60 minutes, which leads to the normal distribution of the active compound inside the tested jar (Figure 21d).

3.7.4.3.5. Thermal release capturing device:

For capturing the active compound release from the tested compound, two protocols were used (Charcoal capturing) and (Cold Column capturing). For Charcoal capturing the device were being set up by using the (Part b) of thermal release device connected with two charcoal column;the first one before the (Part b) for filtering the air and the second after the (Part b) (Thermal generator) for capturing all the active compound releasing from the heated tested material. The cold column capturing is the same principle of charcoal capturing protocol, but instead of using charcoal acetonitrile, 80% was used under the temperature of -20^{o}C. which was provided at room temperature by mixing ice with Ethanol to capture the active compound (Figure 22)

Figure 21: Thermal Releaser; a. exposure part, b. Heating part, c. Air pump, d. Complete thermal releaser device. e. thermometer.

3.7.5. Lethal concentration (LC_{90} and LC_{50}) determination

Determination of the lethal concentration at which 95% of death were obtained (LC_{90}) is based on the mean of mortality values. The mean mortality calculated in each trial was obtained by comparing the percentage mortality due to (crude extract, essential oil, or thermal release) treatment to the percentage of mortality in the DMSO as control. Mortality % = [(Mortality % in treatment - mortality % in control) / (100 - mortality % in control)] x 100

Figure 22: Volatile compound collector; a. Deep freezer -20, b. liquid collector tube, c. Soled (charcoal) collector tube and air filter, d. Complete Volatile collector device.

3.8. Chemical Profiling

3.8.1. Plant Sample preparation

The plant materials used during this study (Seed, Root, and Hairy root) of *H. niger* and *S. indicum*, were prepared as described in section (4), also with the hairy root culture from the bioreactor; moreover the hairy root exudate from the bioreactor and flask culture in three replicates were been used and kept at -20 until chemical analysis.

3.8.2. Bioreactor

3.8.2.1. Hairy roots and hairy root exudates collection

The hairy roots were separated from the medium, blotted dry with sterilized filter paper, and the fresh weight was determined. and the normal plant root was executed from the mature plant and lyophilized (Figure 23), then they are all the samples were kept at -20°C overnight, lyophilized for 72 hours, and the dry weight was determined. The growth medium was cleared by centrifugation for 10 min at 2,900x g to remove debris and stored at -20°C. The media were used for HPLC-ELSD analysis without further purification.

Figure 23: *H. niger* and *S. indicum* Roots. The roots of the mature plant were separated from the aerial part, washed and prepared for lyophilization

3.8.2.2. Analysis of hairy root exudates by HPLC-ELSD

Three replicates were prepared, and the fresh culture medium was used as a blank. The samples were analyzed by HPLC coupled to an Agilent 1260 evaporative light-scattering detector (ELSD) using an Agilent 1290 Infinity II system.

ELSD's temperature for evaporation was set to 42°C, the temperature of the nebulizer was set to 40°C and its flow for nebulizer N2 was 1.6 L/min. A C18 Ether column of Varian Polaris (3 µm particle size, 2 x 100 mm) was used for separation. The sitting of the column temperature was 35°C, and 40 µL of the samples were injected.

HPLC-MS grade methanol obtained from Chemsolute (Germany) and the distilled water purified by Pro-water purification system Arium (Göttingen, Germany) and acidified with 0.1% HPLC-MS grade formic acid (Bucharest, Romania) then used for gradient elution from 2% to 90% water at a flow rate of 0.2 mL/min in 15 minutes.

3.8.3. Analysis of Lignans from hairy root exudates by HPLC-MS

3.8.3.1. Chemicals

Cyclohexane, ethanol, and methanol of HPLC grade were purchased from Sigma Chemical Co., Germany. The lignan standard, sesaminol, was purchased from GERBU Biotechnik GmbH, and (+)-Pinoresinol, Sesamin, Sesamolin, and 3,4-(Methylenedioxy)-phenol (Sesamol) were purchased from Sigma Chemical Co., Germany.

3.8.3.2. Sample preparation

Sample preparation was carried out according to Rangkadilok, with some modifications. Briefly, 1 g of each sample was ground to a fine powder using electric grinding then the seed was weighed accurately and dissolved in 13 mL of 80% ethanol The mix was shaken for 12 hrs at 200 rpm/min. Aliquot of 1000 microliter of the vortex-mixed samples were taken and mixed with 1000 microliter of cyclohexane and defatted for 20 minutes, followed by centrifuging for 5 min at 10,000 rpm/min. The lower phase, which contains the lignans, was transferred into a 2 mL volumetric tube, and the supernatant was discarded. The sample was dried using a speed vacuum before HPLC analysis. On the day of analysis, the sample was re-dissolved in 50% MeOH. (Rangkadilok *et al.*, 2010).

3.8.3.3. HPLC-MS

HPLC-MS grade methanol was obtained from Chemsolute (Th. Geyer, Germany). Distilled water was further purified by an Arium pro ultrapure water system (Sartorius, Germany). Water and methanol were acidified with 0.1% HPLC-MS grade formic acid (Fluka, Germany).

A Phenomenex Synergi Hydro RP column, 50x2 mm with 2.5 µm particle size, was employed. A linear gradient from 5 to 98% methanol in 10 minutes was run, 98% MeOH held for 1.5 minutes, and the column was equilibrated for 5 minutes. An Agilent 1290 Infinity II HPLC system was coupled to an Agilent 6460 triple quadrupole. For sesamol, the $(M+H)^+$ was fragmented, pinoresinol and sesaminol had $(M-H)^-$ parent ions, the sesamolin parent ion was m/z 233 (cleavable ether bond as reported by Takahashi), and the dehydrated of sesamin at m/z 337 was selected as a precursor. Sesmolin and sesamin were both measured in positive ionization mode. (Takahashi *et al.*, 2016).

3.9. Genetic diversity

3.9.1. Plant material

The plant material (lyophilized leaves) used during this study consisted of 25 varieties of *Sesamum indicum* and seven varieties of *Hyoscyamus niger* different. The details of the 32 varieties were listed in section (4).

Leaf material of 32 varieties was collected and used for molecular analysis. The collected plant material from each sample was lyophilized then immediately placed in a 50 mL plastic tube covered with a tight screw cap and kept in -20^{o}C until DNA extraction.

3.9.2. DNA extraction

Total genomic DNA was extracted from seven lyophilized varieties of *Hyoscyamus niger* and 25 lyophilized varieties of *Sesamum indicum* leaf material; the plant leaf (30 mg) placed in 2 m1 Eppendorf-tubes (Figure 24) with 3-5 Wolframcarbide balls and the material ground for 2 minutes (depends on the material) by maximum speed. DNA Extraction started by centrifuging the lyophilized plant leaf shortly (20 sec/8000rpm) then the pulverized material was collected in the bottom of the tubes. A solution which composed of 1mL CTAB-buffer (7,4448 g Na-EDTA (or 50 mL 0,4 M stock solution); 10 g N-Laurylsarcosine; 23 g Sorbitol; 8 g CTAB; 47 g Sodium Chloride; 10 g Polyvinylpyrrolidon 25.000 - 40.000; 800 mL water, pH to 8.0; 10mL

1 M Tris stock solution), 2 µl EtSH (Mercaptoethanol) and 1 µL Proteinase K (Stock solution 20 mg/mL in TE buffer) were added to each sample then incubated at 42°C and 65°C for 10 mins each, respectively. Each sample washed by cold 0.8 mL chloroform/isoamyl alcohol (24:1) for 10mins at 8.000 rpm. 600µl of the upper layer was transferred to a new tube and precipitated with 200 µL PEG 6000 (30%) ((end concentration 6.67%) and 100µl NaCI 5M). The mixture incubated at room temperature for 20 min, followed by 15 min centrifugation at 14000 rpm. The supernatant discarded, and the pill dissolved in 500µl 80% Ethanol and centrifuged 15 min at 14000 rpm. The pellets dried in the Speedvac at 30°C (10-15 min) or under the sterile bench (>2h), and the DNA dissolved in 100µI TE-buffer (10 mM Tris. 1 mM EDTA, pH 8.0). The samples incubated at room temperature for 15-30min and stored in the fridge until checked the quality and the quantity by the gel.

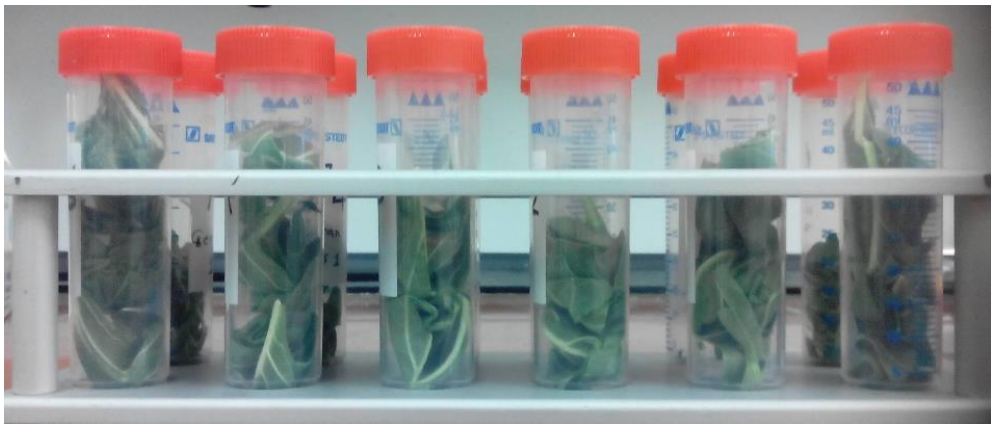

Figure 24: Lyophilized leaves of *Hyoscyamus niger* and *Sesamum indicum*

3.9.3. Primers used for PCR and Sequencing

The specific primers for amplification of *H. niger and S. indicum* were selected from previous reports (Mondal *et al.*, 2016; Park *et al.*, 2014; and some designed kindly by MS. Ling Su). The primers synthesized by Macrogene (Korea) and optimized with gradient PCR. Each primer synthesized different lengths of the fragment, as shown in (Table 4). The specificity of all primers checked using the BLAST search to minimize the non-specific priming and amplification of the homology genes.

Table 4: List of primers used for sequencing.

P. Species	Primer Name	Sequence 5'-3'	Product size	Annealing Temperature	Reference
S. indicum	*FAD2-2F*	AAAAAGACAGATTCCGGTGTGTGG	647	55	Mondal et al. (2016): Plant Genetic Resources: Characterization and Utilization 14:81-90
	FAD2-2R	AATAGGAGATTCCAGATTGCCACC			
	SAD-F	TCCCACGGGAACACAGCTCG	428	59	
	SAD-R	CGTGCATGTGCTCGTTCCTCC			
	O3FAD-1F	GGGTTTTATCAGAATGTGGTCTGAGG	782	57	
	O3FAD-1R	GAAATGAGATCCTTGTTTCCCAGG			
	GBssr-sa-108F	CCACTCAAAATTTTCACTAAGAA	183	61	Park et al. (2014): Plant Breed. Biotech. 2: 80-87
	GBssr-sa-108R	TCGTCTTCCTCTCTCCCC			
	GBssr-sa-182F	CCATTGAAAACTGCACACAA	232	55	
	GBssr-sa-182R	TCCACACACAGAGAGCCC			
	GBssr-sa-184F	TCTTGCAATGGGGATCAG	175	55	
	GBssr-sa-184R	CGAACTATAGATAATCACTTGGAA			
H. niger	*HNTR1-F*	TGCGCTCTGAACGTGACAA	659	50-62	NCBI primer design tool
	HNTR1-R	TATGTATCACCACACCTGCATT			
	HNTR2-F	TCTGAACGAGAAGAGTTTATGAAGA	695		
	HNTR2-R	AAGTGATAAGCAGCCTCAAAGT			

3.9.4. PCR Amplification

Amplifications were achieved in 25 µL reactions using 0.13 µL of One-Taq Polymerase, 5 µL 5x One Tag Standard Reaction buffer, 5 µL 2.5 nM dNTP and 10 µM of each primer. Standard cycling PCR settings were shown in (Table 5), the quality of the products was observed on ethidium bromide-stained agarose gels, PCR products were directly sent for sequencing. PCR amplicon for sequencing was generated from a 10 µL genomic DNA template.

3.9.5. DNA Precipitation: Isopropanol

The DNA was precipitated by using 70% Isopropanol and incubated at room temperature for 15 min and centrifuged at maximum speed for 10 mins. The samples were washed by 200 µL 70% Ethanol and centrifuged for 10 mins. The ethanol discarded, and the samples dried in Speed vacuum for 10 mins then the DNA was re-dissolved in Bidest water.

Table 5: Standard cycling PCR settings.

	Step	Initial denaturation	Start Loop 30x Denaturation	Primer Annealing	Extension	Closed Loop	Store at
One Taq *HNTR1* and *HNTR2* PCR running protocol	Temperature	94.0°C	94.0°C	62.0°C	68.0°C	68.0°C	8.0°C
	Time	30 sec	30 sec	1 min	1 min	5 min	10 min 1 sec
One Taq *FAD2* (old) PCR running protocol	Temperature	94.0°C	94.0°C	55.0°C	68.0°C	68.0°C	8.0°C
	Time	30 sec	30 sec	1 min	5 min	5 min	10 min 1 sec
One Taq *SAD* (old) PCR running protocol	Temperature	94.0°C	94.0°C	59.0°C	68.0°C	68.0°C	8.0°C
	Time	30 sec	30 sec	1 min	1 min	5 min	10 min 1 sec
NEB *HNTR1* and *HNTR2* PCR running protocol	Temperature	95.0°C	95.0°C	63.0°C	68.0°C	68.0°C	20°C
	Time	6 min	30 sec	1 min	1 min	5 min	10 min
NEB *SAD* and *FAD* (new) PCR running protocol	Temperature	95.0°C	95.0°C	60.0°C	68.0°C	68.0°C	20°C
	Time	6 min	30 sec	1 min	1 min	5 min	10 min

3.9.6. Analysis of the Amplified DNA Fragments by Gel Electrophoresis

The amplified products analyzed using agarose gel electrophoresis. The gel was prepared using 0.8% (w/v) agarose (Fisher) in 1x TBE buffer (Sigma, pH=8.0). The gel was run in 1x TBE (Tris/ Boric acid/ EDTA) running buffer in an electrophoresis tank (Hoefer Scientific Inc.) after loading an aliquot of the PCR product (2µL) with a loading buffer dye (6x) (Thermo). Diluted samples were loaded in equal volume in independent wells along with a known concentration of DNA as a control. A 1Kb DNA marker was also loaded. The samples run at 60 V for 60 min (Power supply model: 200/2.0/ Bio-Rad). The DNA bands were visualized using a UV light transilluminator. The gels have been removed from the plates using a gel separator after electrophoresis and placed in a bromide solution tank with ethidium for 5 to 10 minutes. These gels were then moved to a visualization gel documentation system. The images were captured and stored for analysis.

3.9.7. Sequencing and Bioinformatics

Amplified PCR products were diluted 1/5 in ddH_2O and sequenced using the Sanger method (Macrogen / Korea). Only one primer was used for sequencing (Table 4). The sequences were analyzed and compared with the reference sequence published in NCBI using MEGA7.

3.10. Statistical analysis

The means ± standard deviation (SD) is calculated where appropriate. Statistical differences were determined by the ANOVA, followed by Dunnet's test and the level of significance set at $P<0.05$ using (GraphPad Prism version 7.1). In many cases, results were calculated as the percentage of relevant control values to make understanding of the results easier.

CHAPTER FOUR

4. RESULTS

4.1. Seed germination

Generally, *H. niger* seeds have a strong seed dormancy even under normal laboratory conditions. thus, the seeds were treated with different gibberellic acid (GA3) concentrations 0.0, 2.5, 25, and 250 mg/L for 12 h at room temperature (22±2ºC) (Figure 25). Interspecifically, the total germination rate significantly differed among the selected species at each GA3 concentrations. The black henbane seeds germination treated with different GA3 concentrations is shown in (Figure 26) and for *Sesamum indicum* is shown in (Figure 27). According to the analysis of the results, different GA concentrations had significant effects on the germination rate ($P \leq 0.05$).

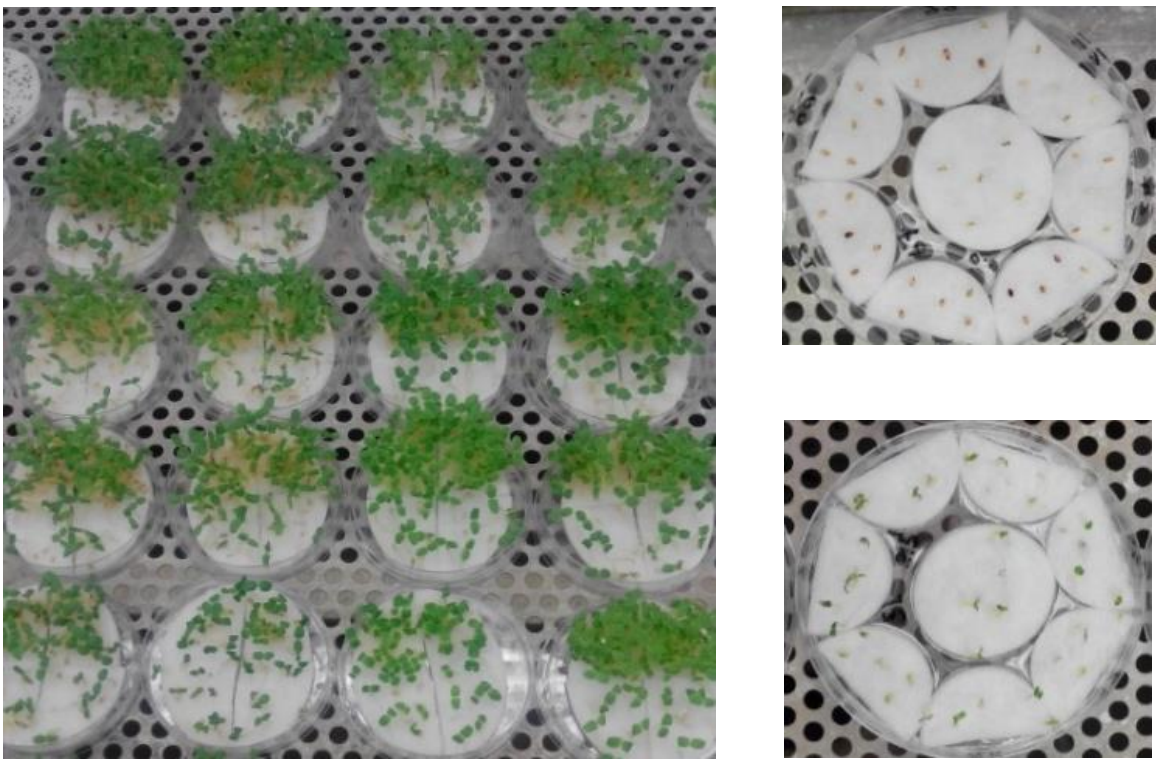

Figure 25: *In vitro* propagation cultures of *Hyoscyamus niger* and *Sesamum Indicum*, Germination of seeds on a sterilized cotton pad after a period of 31 days.

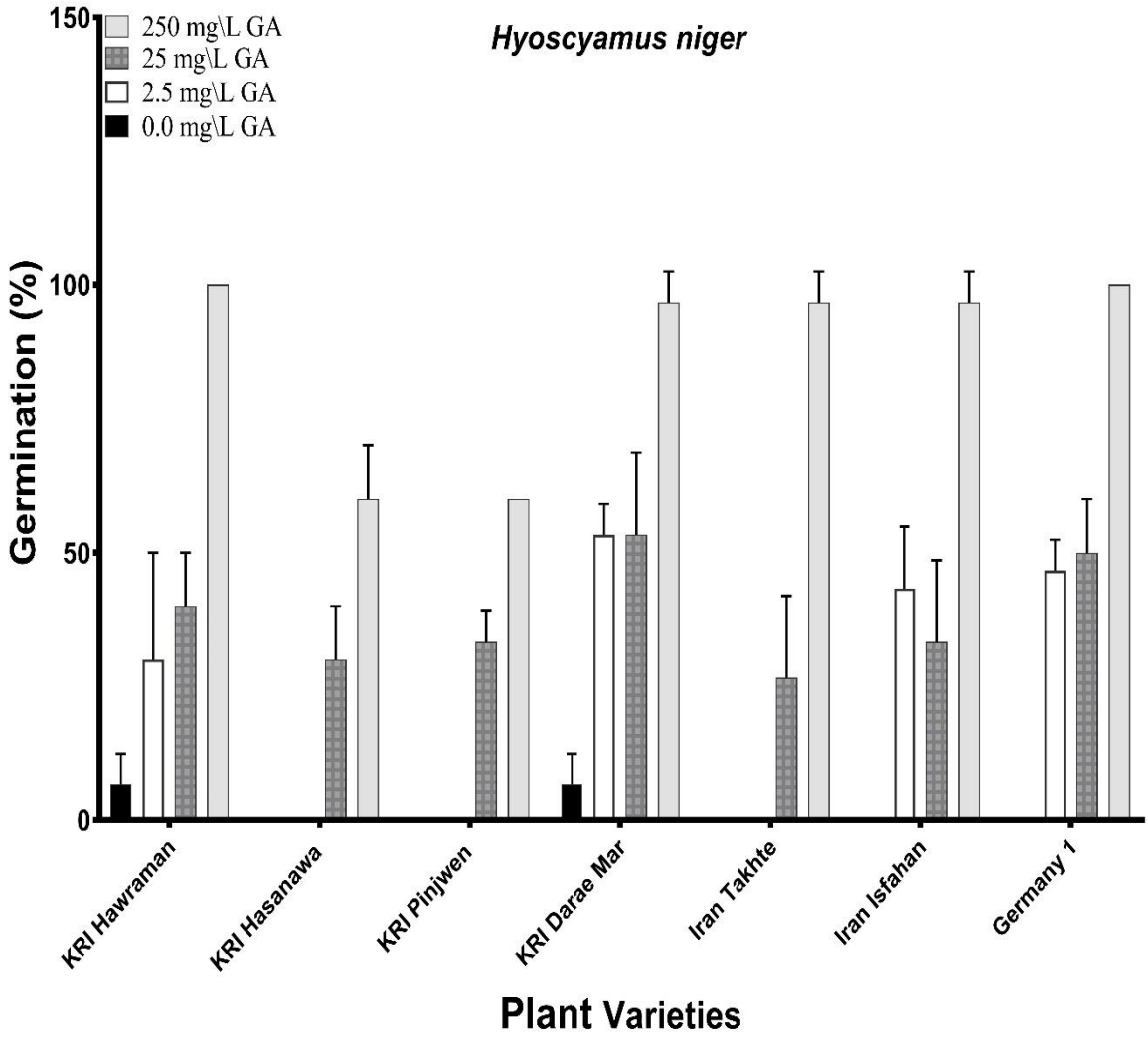

Figure 26: Effects of different concentrations of GA3 on *H. niger in vitro* seed germination

Within pre-soaking treatments, 250 mg/L GA3 treatment gave the highest germination rate with 98-100%. The highest germination rate was obtained from 250 mg/L GA3 treatment compared to the control, which reached (92-95%) significant differences. Germination enhancing effects of different concentrations of GA3 observed in (Figures 25 and 26) which shows the final mean percentage germination after 30 days. The first signs of germination were noted after three days of seeds growing. The type of treatment influenced the day in which the first lot of seeds started to germinate.

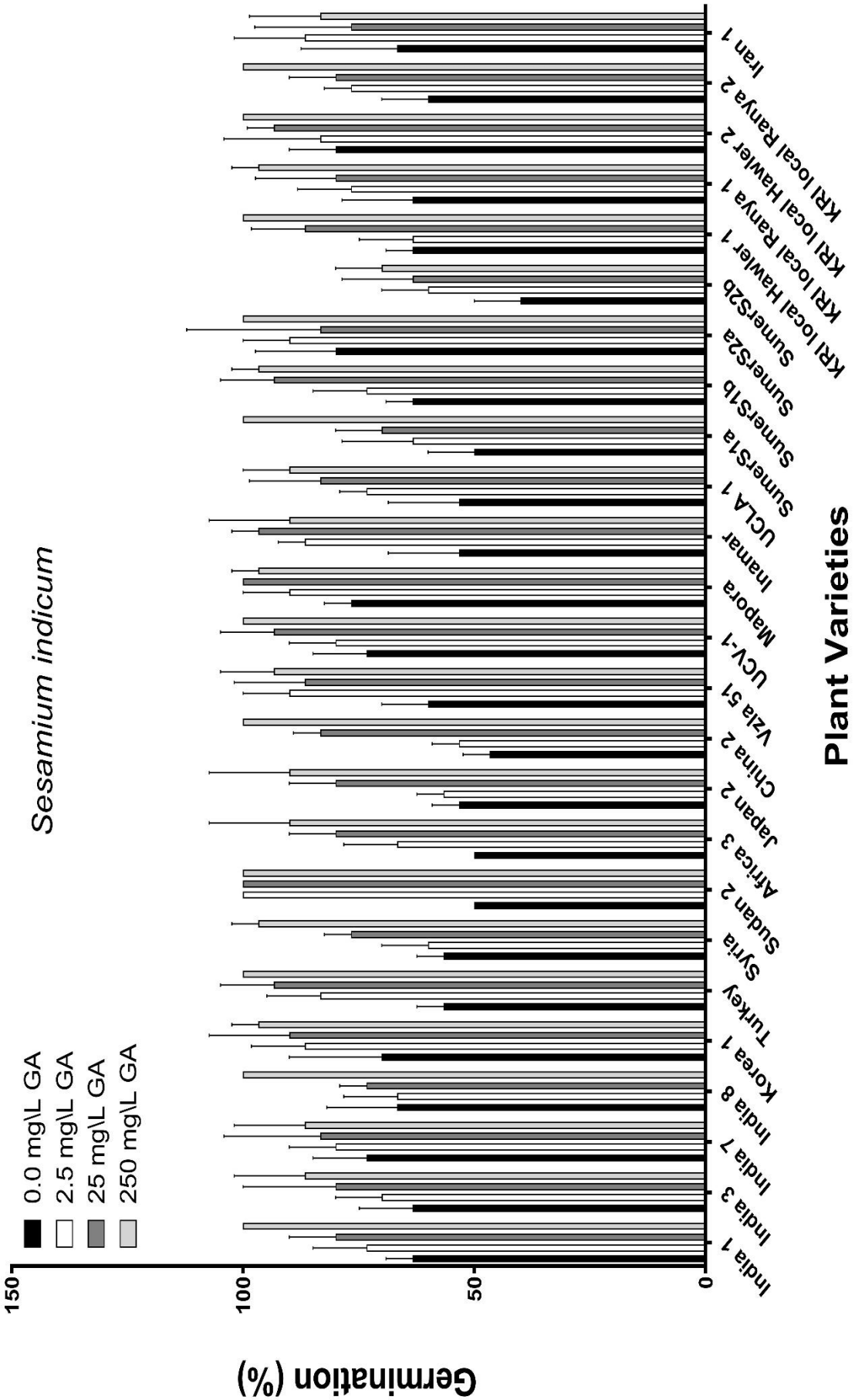

Figure 27: Effects of different concentrations of GA3 on *S. indicum in vitro* seed germination.

4.2. Seed sterilization

After four-weeks of observation, 3% of fungal and bacterial contamination was observed in most seeds. Meanwhile, the leaves' surface sterilization with 70% ethanol and 6% commercial Clorox shows effective decontamination efficiency but significantly affected the survival and regeneration potential.

4.3. Effect of the different sterilization methods on Leaves viability and Hairy Root Induction

Two types of explant sterilization methods were used. The capability of these methods was examined to test the viability of plant leaves from *H. niger* and *S. indicum* hairy root induction. Both methods were efficient and the induction of hairy roots observed with different efficiencies (Figure 28).

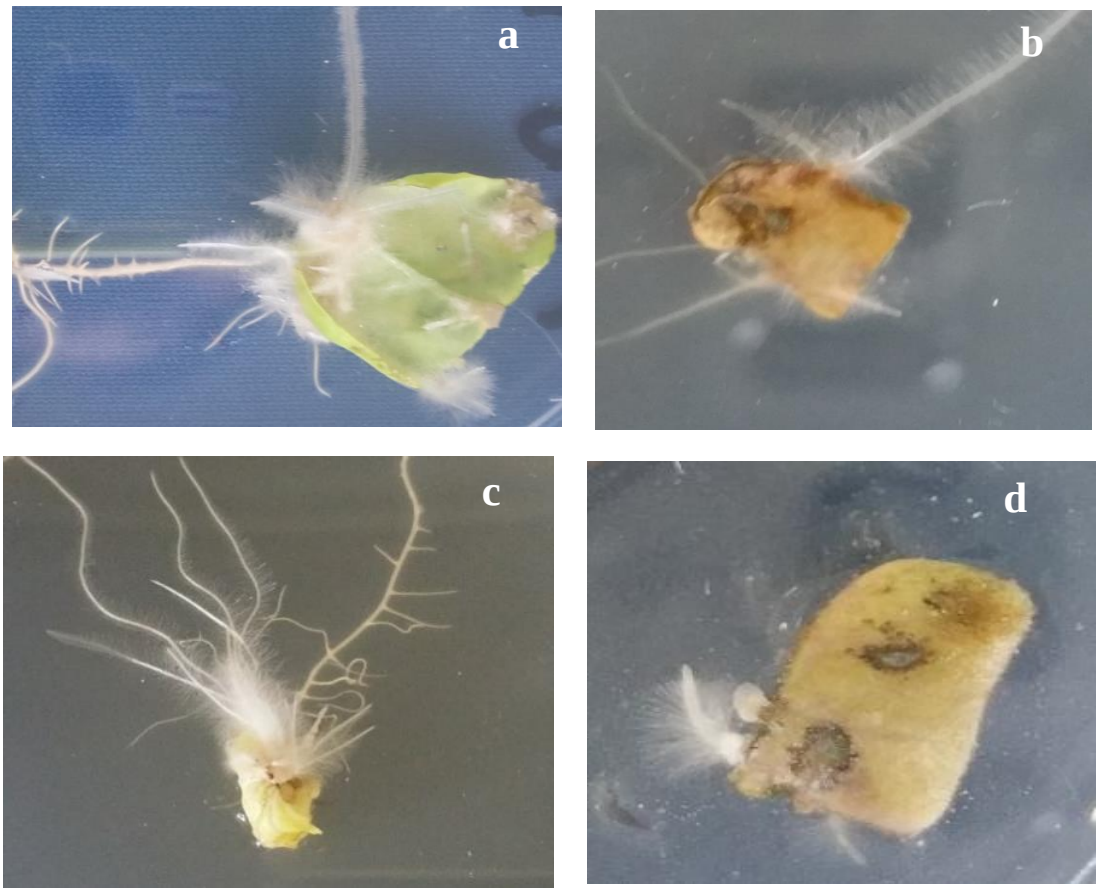

Figure 28: Effect different sterilizations methods on hairy root production in *Hyoscyamus niger* a,b and *Sesamum indicum* c,d, (a,c aseptic growing explant, and b, d no aseptic growing explant)

The best response (Hairy Root Induction%) was achieved by the aseptic growing procedure with the explant initiated from sterilized seed, the highest response was %100 after 1 to 2 weeks of cocultivation in both plant species, while the lowest response was 31.2% in sesame plant with Sumer S2b varieties for the aseptic protocol also lowest contamination was achieved (Figures 29 and 30).

Meanwhile, the non-aseptic protocol in which the explant needs surface sterilization, the highest response was 37.5% in Sesame plant(India7) varieties, while the lowest response was 12.5% in Henbane plant (Germany 1) varieties. This showed that the frequency of hairy root induction was dependent on the sterilization method (Figures 31 and 32). Leaf disks from all plant variates that were not transformed with the *A. rhizogenes* exhibited no hairy root induction.

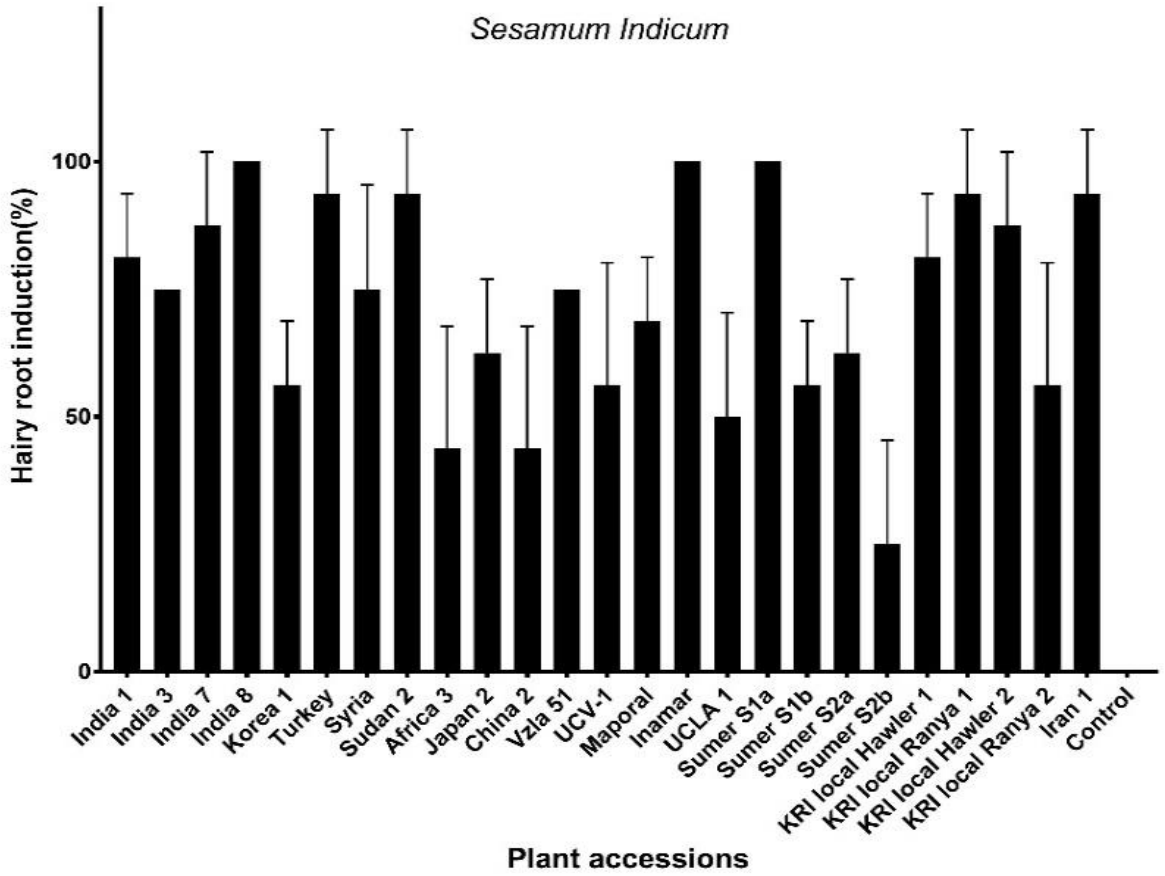

Figure 29: Response of *Sesamum indicum* plant variates to hairy root induction using aseptic condition (n=4)

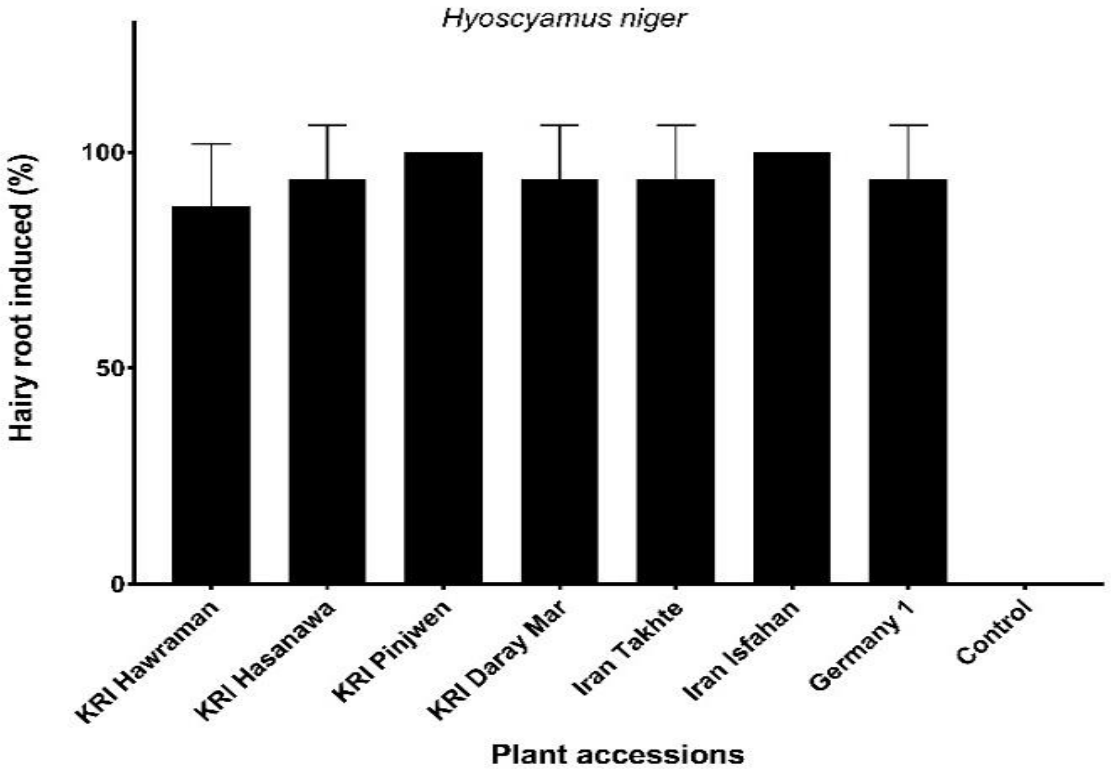

Figure 30: Response of *Hyoscyamus niger* plant variates to hairy root induction using aseptic condition (n=4)

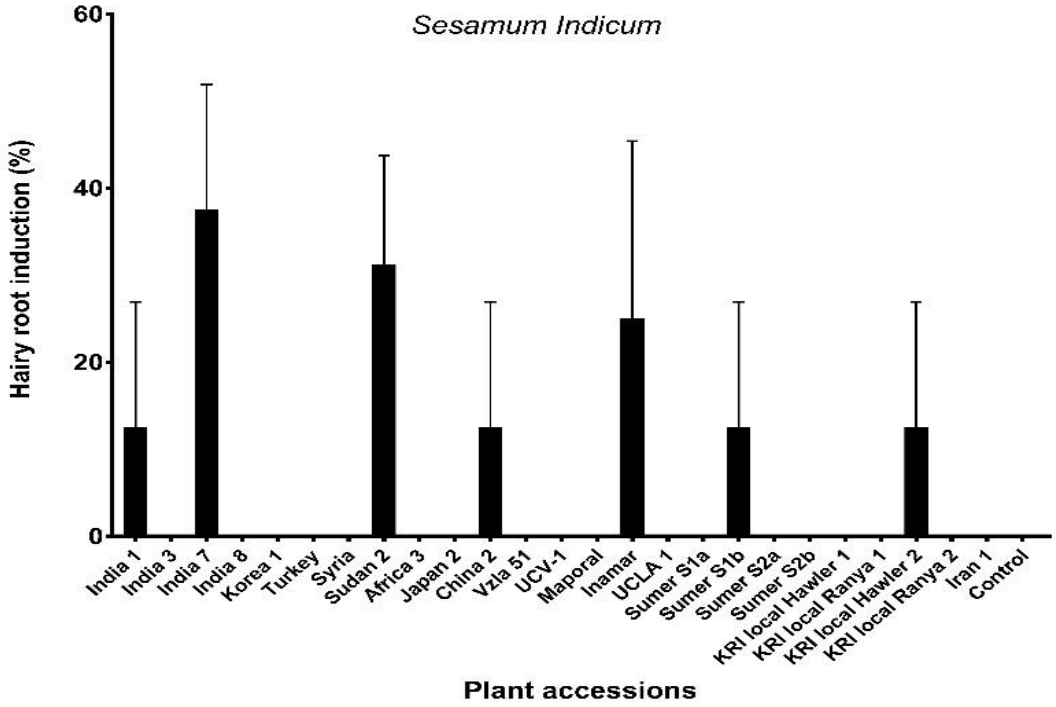

Figure 31: Response of *Sesamum indicum* plant variates to hairy root induction using non-aseptic conditions (n=4)

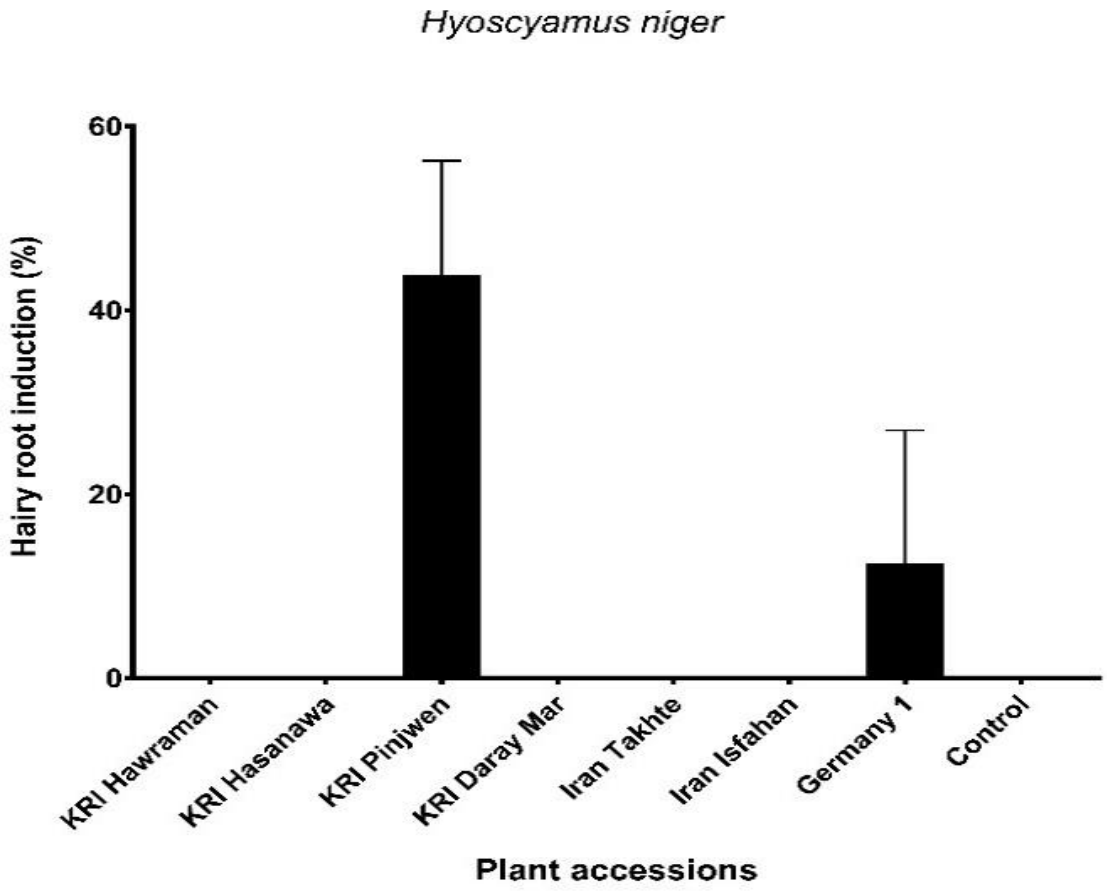

Figure 32: Response of *Hyoscyamus niger* plant variates to hairy root induction using non-aseptic conditions (n=4)

The results showed a significant difference in hairy root induction. Moreover, the viability of the explants also differs in both protocols. The hairy root structure between both plant species and their varieties are also different. In the aseptic plant growing method, the quality of the HRs is more compact with good viability, and the explants remain green fresh looking, while in the non-aseptic plant growing method, the quality of the HRs is less dense with poor viability, and the explants turned brown and died faster.

4.4. Hairy root initiation

4.4.1. Efficacy of Different *A. rhizogenes* Strains on Hairy Root Induction

For hairy roots induction, two strains of *A. rhizogenes* were been tested, ATCCA15835 and A4. The effectiveness of these agrobacterium strains was determined by comparing their ability to induce hairy root on explants. Transformation frequency through *A. rhizogenes* ATCC15835 and *A. rhizogenes* A4 strains were recorded after 1 to 2 weeks of inoculation, and hairy roots appeared from the cut edges of the explants inoculated with these two strains (Figure 33). The transformation frequency was observed in both plant species using *A. rhizogenes* ATCC15835 and A4. The TF using ATCC15835 species on *Hyoscyamus niger* recorded 100%, and the percentage of hairy root induction was 100% for KRI Hawraman and KRI Hasanawa which was 87.5%, compared with *A. rhizogenes A4* in which the TF was 71.4%, and the highest percentage of hairy root induction was 56.25%, and the lowest was 12.5% for KRI Daray Mar varieties while Iran Isfahan and KRI Hawraman did not succeed in hairy root generating (Figures 34, 35, 36, and 37)

Figure 33: Induction of transformed hairy roots after Co-cultivation with different strains of *A. rhizogenes* species ATCC15835 and A4 from *Hyoscyamus niger* and *Sesamum indicum* grown on hormone-free MS media.

Meanwhile, the transformation frequency of *Sesamum indicum* was 100% using the ATCC15835 strain. The explants from only two varieties, Sudan 2 and Sumer S1a, reached 100% hairy root induction, and the lowest induction was 31.25%, compared with *A. rhizogenes* A4, in which the TF was 68%, and the highest hairy root induction was 56.25%, and the lowest was 12.5% for the six varieties while eight varieties did not generate hairy root (Figure 38). All the varieties of both plant species show significant hairy root induction using *A. rhizogenes*; ATCC15835, while only 13 varieties of both plant species show significant results using *A. rhizogenes* A4. Different response of different plant different species and varieties of the same genus towards the same strain of *A. rhizogenes* has been noted to a great extent (Sharafi *et al.*, 2012). Such difference of response in plants towards the same *A. rhizogenes* strain could be attributed to the differential expression of the endogenous hormonal level of the host genotypes towards the integrated Ri T-DNA (Ryder *et al.*, 1985).

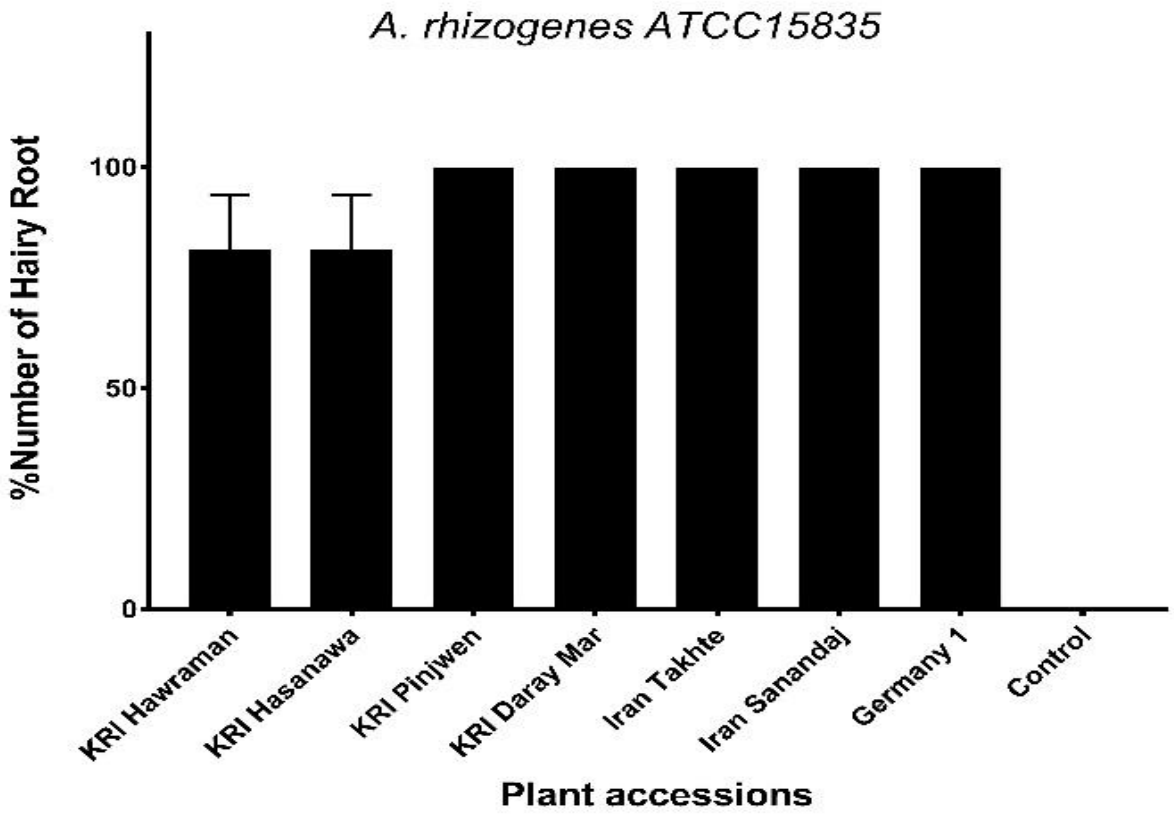

Figure 34: Hairy root initiation on *Hyoscyamus niger* varieties using *A. rhizogenes* ATCC15835, on Semi-solid MS medium.

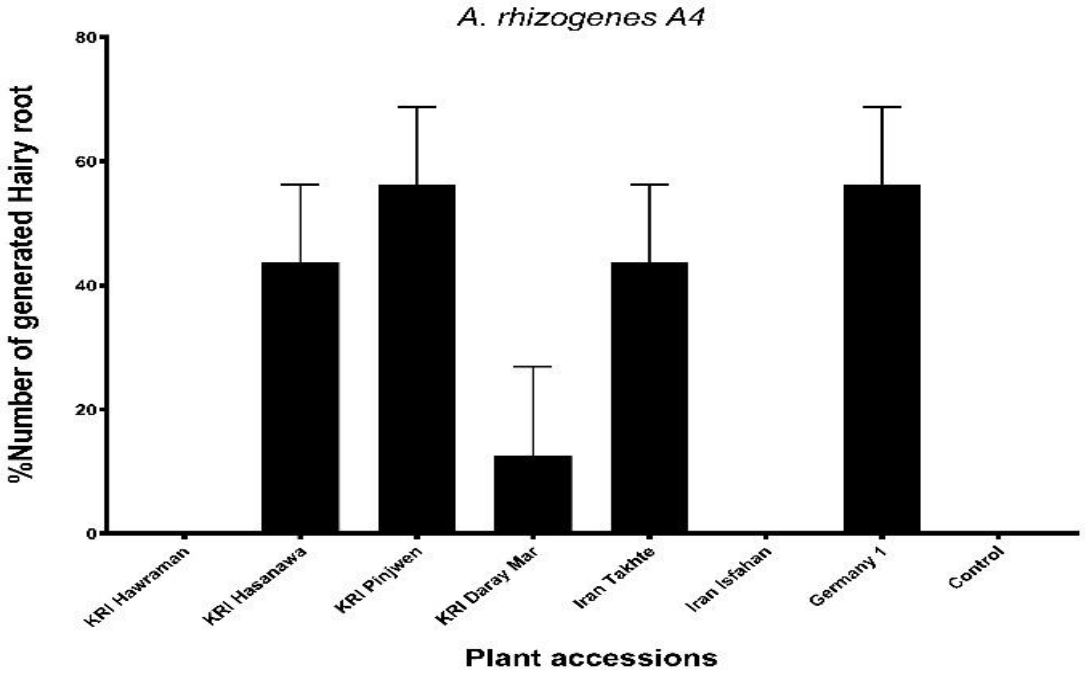

Figure 35: Hairy root initiation on *Hyoscyamus niger* varieties using *A. rhizogenes* A4 on Semi-solid MS medium.

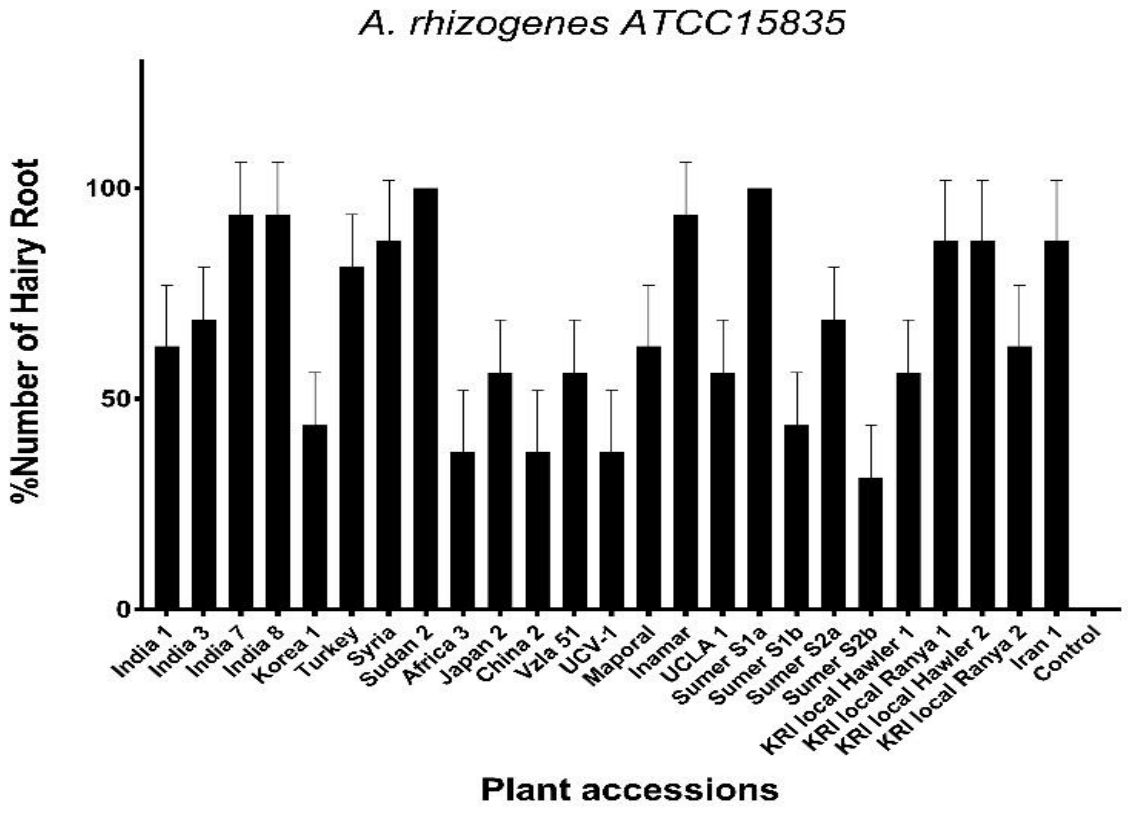

Figure 36: Hairy root initiation on *Sesamum indicum* using *A. rhizogenes* ATCC15835, on Semi-solid MS medium.

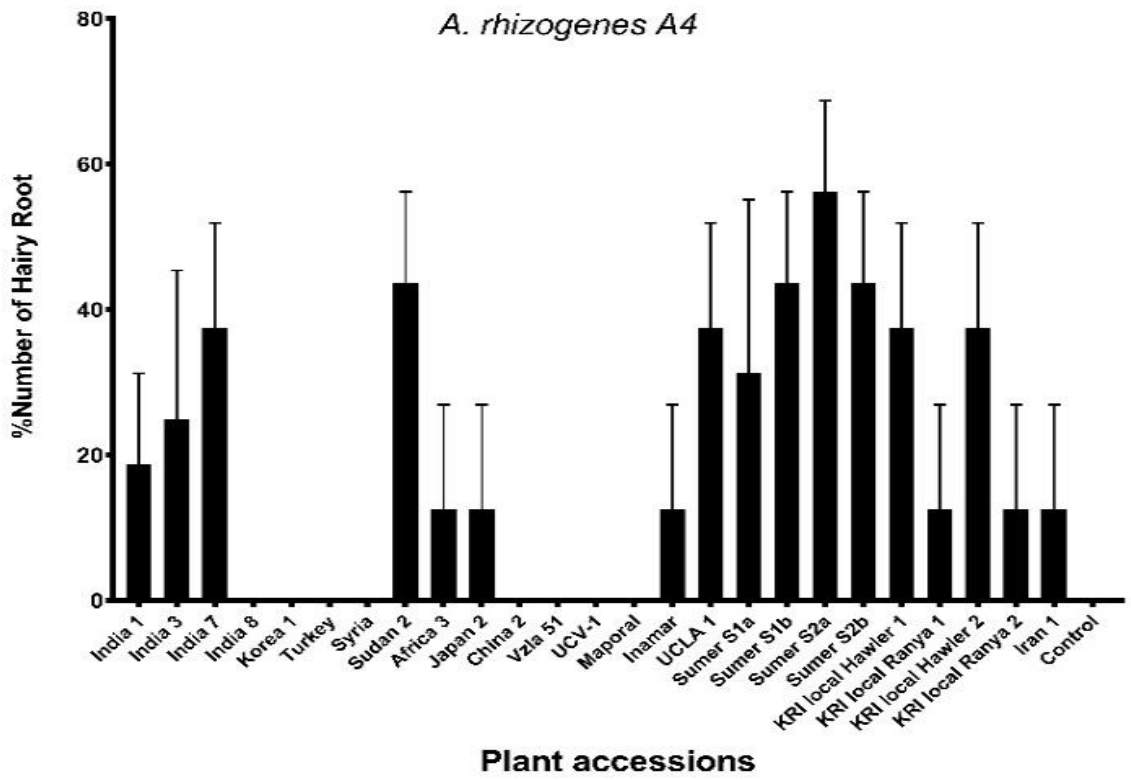

Figure 37: Hairy root initiation on *Sesamum indicum* using *A. rhizogenes* A4, on Semi-solid MS medium.

None of the control explants from both plant species under identical *in vitro* condition has demonstrated a sign of hairy root generation during prolonged culture of incubation. Hairy roots developed on the explants and did not necessarily follow the laws of geotropism but grew horizontally and also upwards (Figure 38). This appears to be a distinct characteristic that separates them from normal roots. The points of infection turned black or dark, and this was probably due to the phenolic compounds that were produced by the explant as they formed as defense mechanisms against the bacterial infection. To improve the efficiency of hairy root induction, a range of bacterial concentrations (OD600nm=1.0 and 1.3) with inoculation time of 5 minutes were tested. The results showed that the best hairy root induction % occurred with OD600 equal to 1.3, and when inoculated for 5 minutes. The statistical analysis showed significant differences in hairy root induction% between both plant species and their varieties using *A. rhizogenes* bacterial Strain ATCC15835 and A4. The results showed a significant difference in the percentage of hairy root induction and transformation frequency regarding both bacterial strains.

A. rhizogenes ATCC15835 produced hairy roots in the shortest time (9 Day), and it had the highest transformation efficiency (100%). While the strain A4 showed lower transformation frequency. This confirms that hairy roots only developed at the specific sites of infection. It was noted that once the hairy roots got into contact with bacterial mass, they die since the bacterial growth overwhelmed the hairy roots (Figures 38).

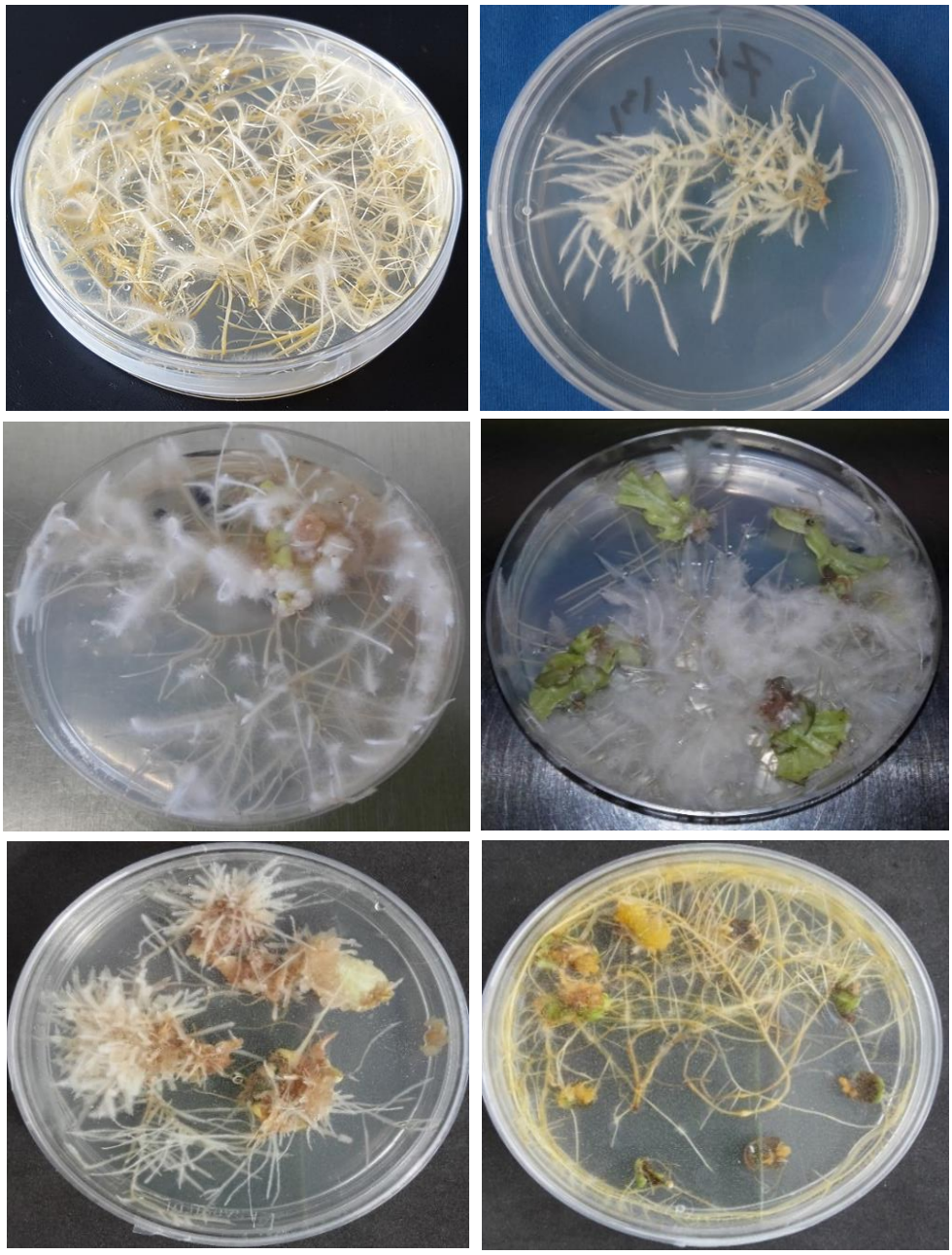

Figure 38: Hairy roots induced on *Hyoscyamus niger* and *Sesamum indicum* and their varieties using *A. rhizogenes* ATCC15835, and A4 with anti-gravitropic root mass growing on solid medium after 30 days of culture.

4.4.2. Hairy Root Growing in Liquid Medium

The Hairy root clones from both plants were inoculated into cefotaxime-containing medium to kill *A. rhizogenes* bacteria. These roots were subcultured at least three times on this medium until the roots were free of bacteria growth and ready to use as a clone for the HR liquid culture medium (Figure 39). The hairy root induction frequency using *A. rhizogenes* ATCC15835 for Henbane and Sesame was 100% while using *A. rhizogenes* A4 for Henbame was 28.5% and for Sesame 56.0%.

During cultivation, the hairy roots inoculated on the MS liquid medium showed typical hairy root characteristics such as the ability to grow in Plant growth regulator hormone-free media as well a typical anti-gravitropic growth (Srivastava and Srivastava, 2007, Veena and Taylor, 2007) (Figure 40).

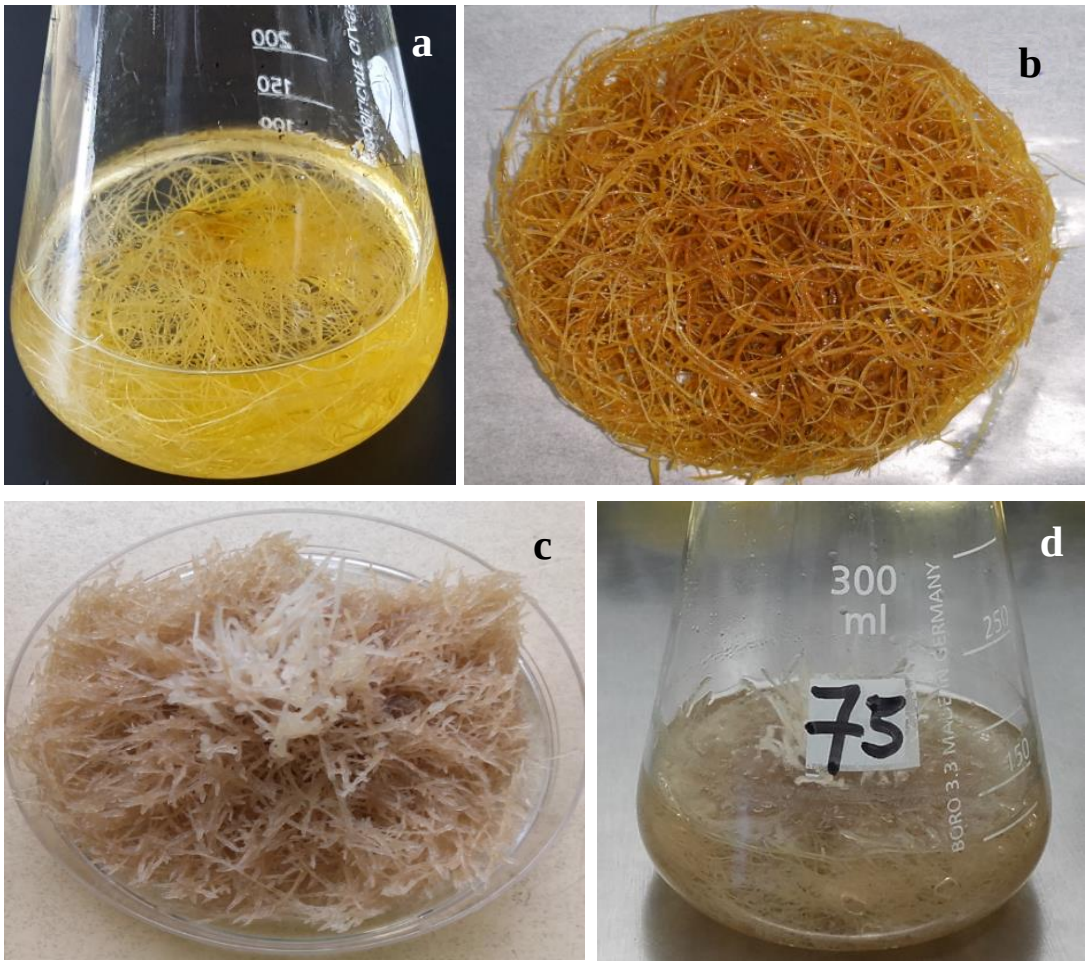

Figure 39: Hairy roots cultured in solid and liquid cultures displaying a typical hairy root response. Liquid-shake culture of clone *Hyoscyamus niger* (c,d)and *Sesamum indicum* (a,b) showing abundant proliferation of hairy roots. Cultures were grown for 30 days.

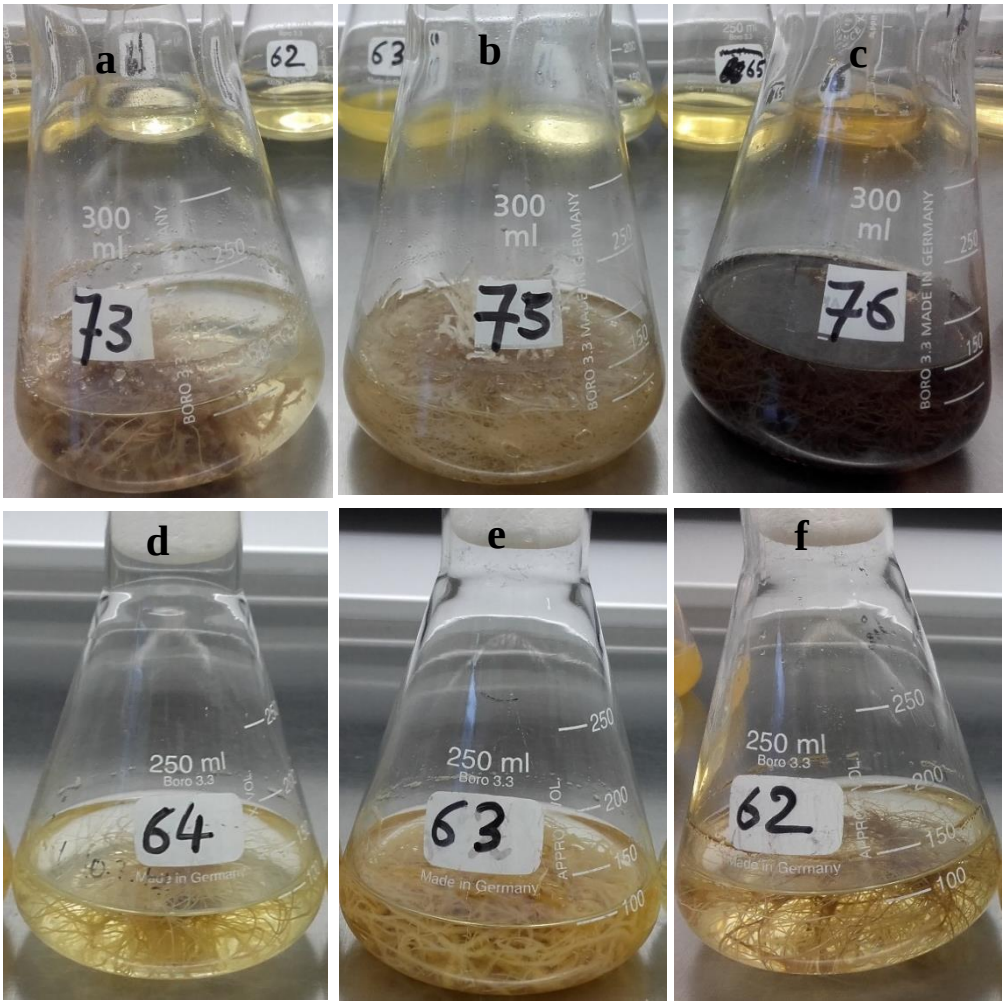

Figure 40: Liquid cultures of the hairy roots of *Hyoscyamus niger* (a, b, c) and *Sesamum indicum* (d, e, f) variates cultivated on MS liquid medium displaying differences in their growth and morphology patterns

The growth pattern of the hairy root was not uniform, and it has been observed that the growth was different in each plant varieties, regarding biomass production. The maximum biomass yield was observed on the 30th day for *H. niger* and *S. Indicum*. The Hairy root biomass yield on *S. indicum* was presented in (Figure 41) and *H. niger* (Figure 42) using *A. rhizogenes* ATCC15835 and A4 strain, in liquid MS medium. The highest biomass yield for *H. niger* using *A. rhizogenes* ATCC15835 and A4 strain was 10.2 g/L and 7.4 g/L respectively, while biomass yield on *S. indicum* using *A. rhizogenes* ATCC15835 and A4 strain were 9.0 g/L and 6.6 g/L respectively.

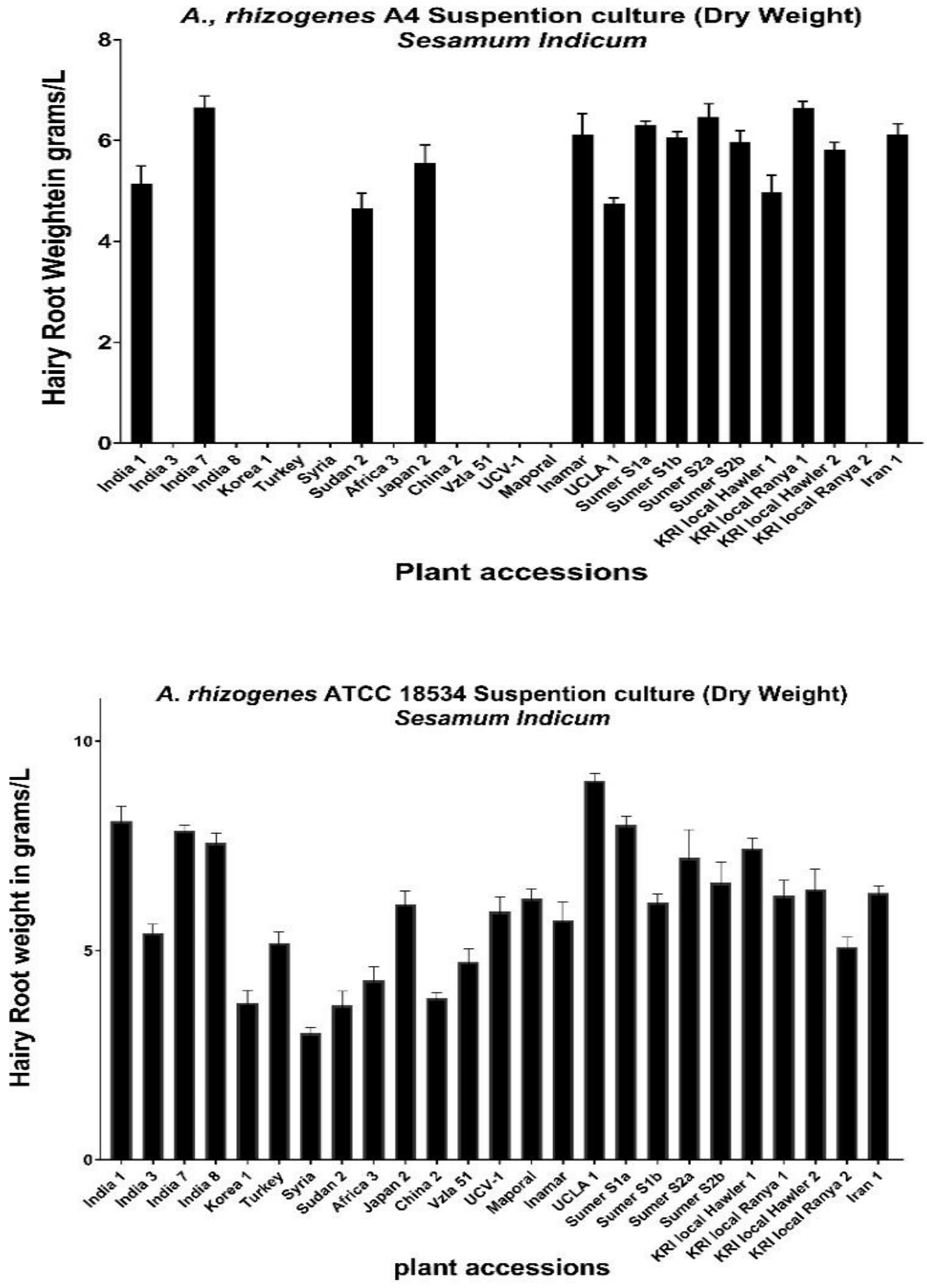

Figure 41: Hairy root initiation on *Sesamum indicum* leaf explant using *A. rhizogenes* ATCC15835 and A4 strain, grown in a liquid MS medium.

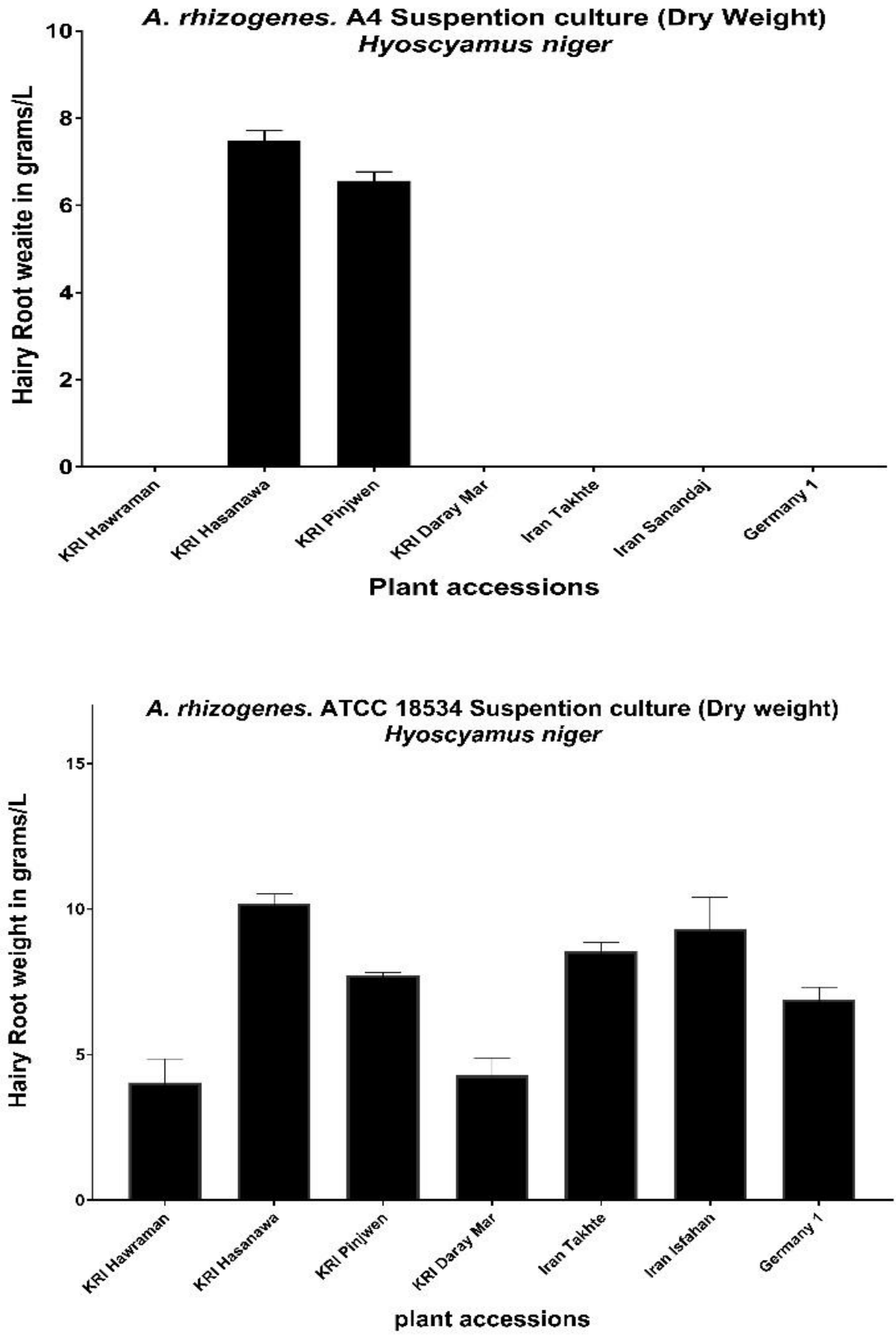

Figure 42: Hairy root initiation on *Hyoscyamus niger* leaf explant using *A. rhizogenes* ATCC15835 and A4 strain, grown in a liquid MS medium.

The biomass production and the morphological structure of the hairy root are significantly different between both plant species *H. niger* and *S. indicum* by using both *A. rhizogenes* strain ATCC18534 and A4 regarding both plant species. The active cell division zone located in the root tips remains highly active during the root growth, and at this stage, the cell utilizes the medium components as well as exogenously supplied chemical compounds. Previous studies showed that bioconversion occurred more efficiently, demonstrating that pre-cultured roots had time to increase biomass and thus raise the number of biologically active cells (Hakkinen *et al.*, 2012). The hairy root dry and fresh weights of the biomass are significantly different between both plant varieties. Moreover, some of the varieties from both plant species showed morphological different hairy root structures (Figure 43).

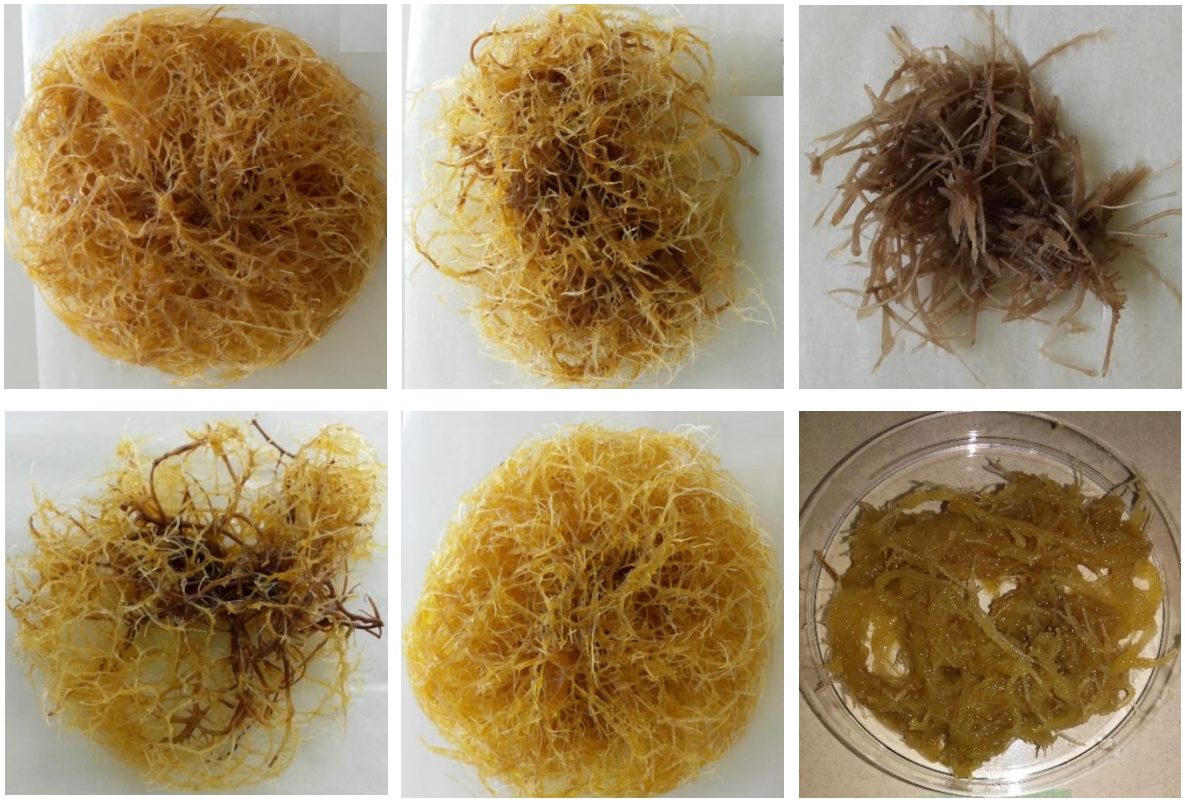

Figure 43: Fresh hairy root biomass is grown in liquid MS medium, Showing differences in the quality, texture, and morphology of the hairy root.

4.5. Bioreactor

4.5.1. Hairy-root biomass yield in flask cultures and bioreactor

Bubble Bioreactor is one of the most common mass propagating reactors for various hairy root cultures (Figure 44). It is shown in (Table 6) that the highest dry weight biomass obtained in this bioreactor was (13.70 g/L) for *Hyoscyamus niger* and (12.10 gr/L) for *Sesamum indicum*, which was significantly different compared with the flask hairy root culture (Hairy root grown in conical flask with flask air and agitation) which was (9.31 gr/L) for *H. niger* and (7.58 g/L) for *S. indicum* a highest biomass recorded (Figure 45).

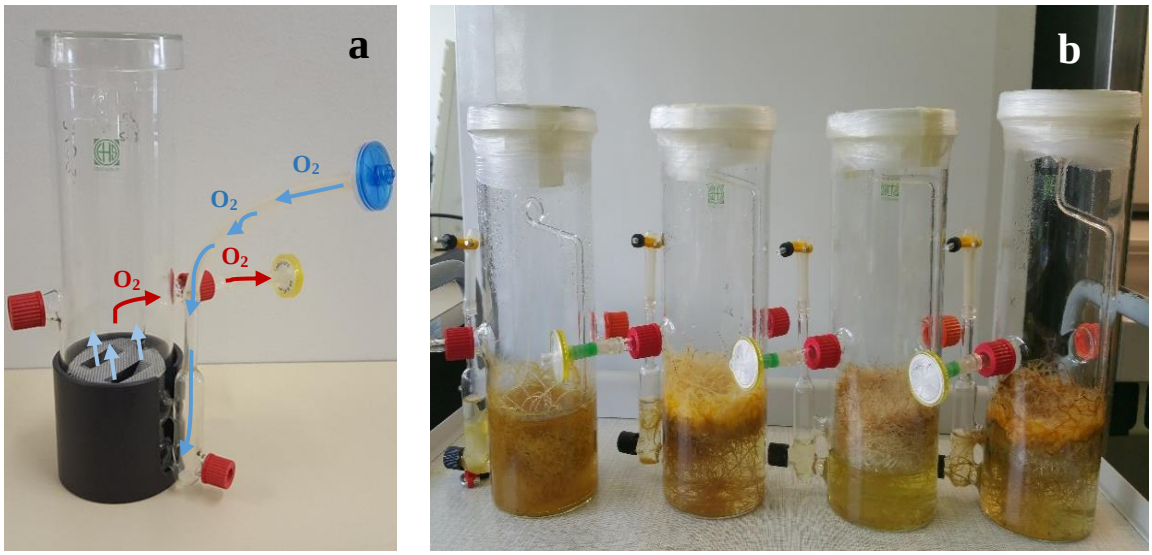

Figure 44: Cultivation of hairy root culture in a Bubble reactor. (A) Bioreactor set-up; (B) Hairy roots obtained after 30 days of growth.

The hairy roots grew in flask culture hypoxic stress, which related to poor aeration and less oxygen solubility in the culture medium as compared to reactors like Bubble column reactor. This was due to the low saturation concentration of DO (dissolved oxygen) in water generally. The result shows that the biomass yield in the bioreactor is significantly different and higher compared to the flask culture, except (Japan 2, and Sumer S2b) varieties with no significant differences between them.

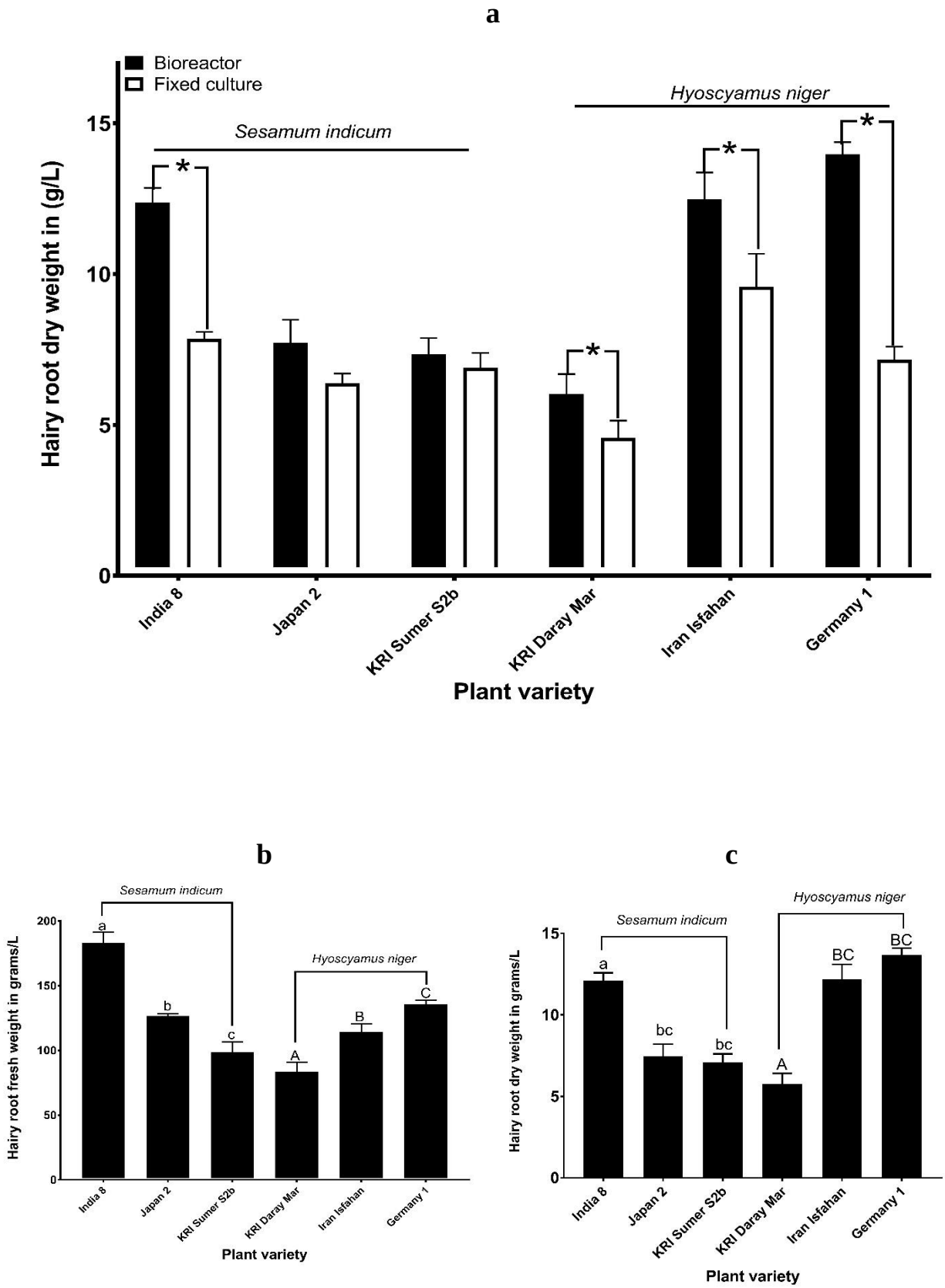

Figure 45: Hairy root initiation from *Hyoscyamus niger* and *Sesamum indicum using*; a. Comparison of biomass yield in dry weight between the bioreactor and flask culture. b. Fresh hairy root biomass from bubble bioreactor. c. Dry, hairy root biomass from bubble bioreactor. Bars with different litter are significantly different P≤ 0.01.

Table 6: Hairy root biomass (g/L) dry weight of three *Hyoscyamus niger* and *Sesamum indicum* verities using bioreactor and flask culture. The means followed by the same letter are not significantly different according to Duncan multiple range tests (P≤ 0.01)

Plant Varieties	Bioreactor		Flask culture	
	Hairy root g/L ±SD	CI 99%	Hairy root g/L ±SD	CI 99%
Sesame India 8	12.10 ± 0.48 **a**	11.38 – 12.81	7.57 ± 0.23 **b**	7.23 – 7.91
Sesame Japan 2	7.44 ± 0.77 **b**	6.29 – 8.58	6.11 ± 0.31 **c**	5.65 – 6.57
Sesame Sumer S2b	7.07 ± 0.53 **b**	6.28 – 7.86	6.62 ± 0.48 **c**	5.91 – 7.33
Henbane KRI Daray Mar	5.15 ± 65 **c**	4.18 – 6.12	4.28 ± 0.58 **d**	3.42 – 5.14
Henbane Iran Isfahan	12.19 ± 0.89 **a**	10.86 – 13.51	9.31 ± 1.09 **a**	7.69 – 10.93
Henbane Germany 1	13.69 ± 0.14 **a**	13.07 – 14.30	6.98 ± 0.43 **bc**	6.25 – 7.53

Hairy root cultures were established from a primary hairy root clone, using flask culture and bubble bioreactor for biomass accumulation for both plant varieties *Hyoscyamus niger* and *Sesamum indicum* (Figure 46). The bioreactor biomass of hairy root cultures after 30 days ranged between 13.7 g and 5.0g dry weight per1000 mL (Figure 45). From the data reviewed in (Table 7), it was observed that the fresh and dry weight biomass is significantly different within the varieties of both species and also between both plant species. Comparing the bioreactor with the flask air culture, the overall biomass was higher in the bubble bioreactor (Kim *et al.*, 2002).

Table 7: Means of the dry weight of *Hyoscyamus niger* and *Sesamum indicum* culture, in bubble bioreactor and flask cultures.

Plant Varieties	Bioreactor		Flask culture	
	Mean	SD	Mean	SD
India 8	12.10	0.48	7.57	0.23
Japan 2	7.44	0.77	6.11	0.31
KRI Sumer	7.07	0.53	6.62	0.48
KRI Daray	5.75	0.65	4.28	0.58
Iran Sanandaj	12.19	0.89	9.31	1.09
Germany 1	13.69	0.41	6.89	0.43

This study indicates that using a bioreactor with agitation that achieved by the air bubbles rising through the medium and a plastic net with the glass stand was used to fix the cultures, enables greater biomass production comparing with flask culture with flask aeration. The production of the vulnerable secondary metabolites is highly on demand. Therefore, it is required to design a suitable bioreactor that can generate high biomass of hairy root without decreasing the quantity of the secondary metabolite.

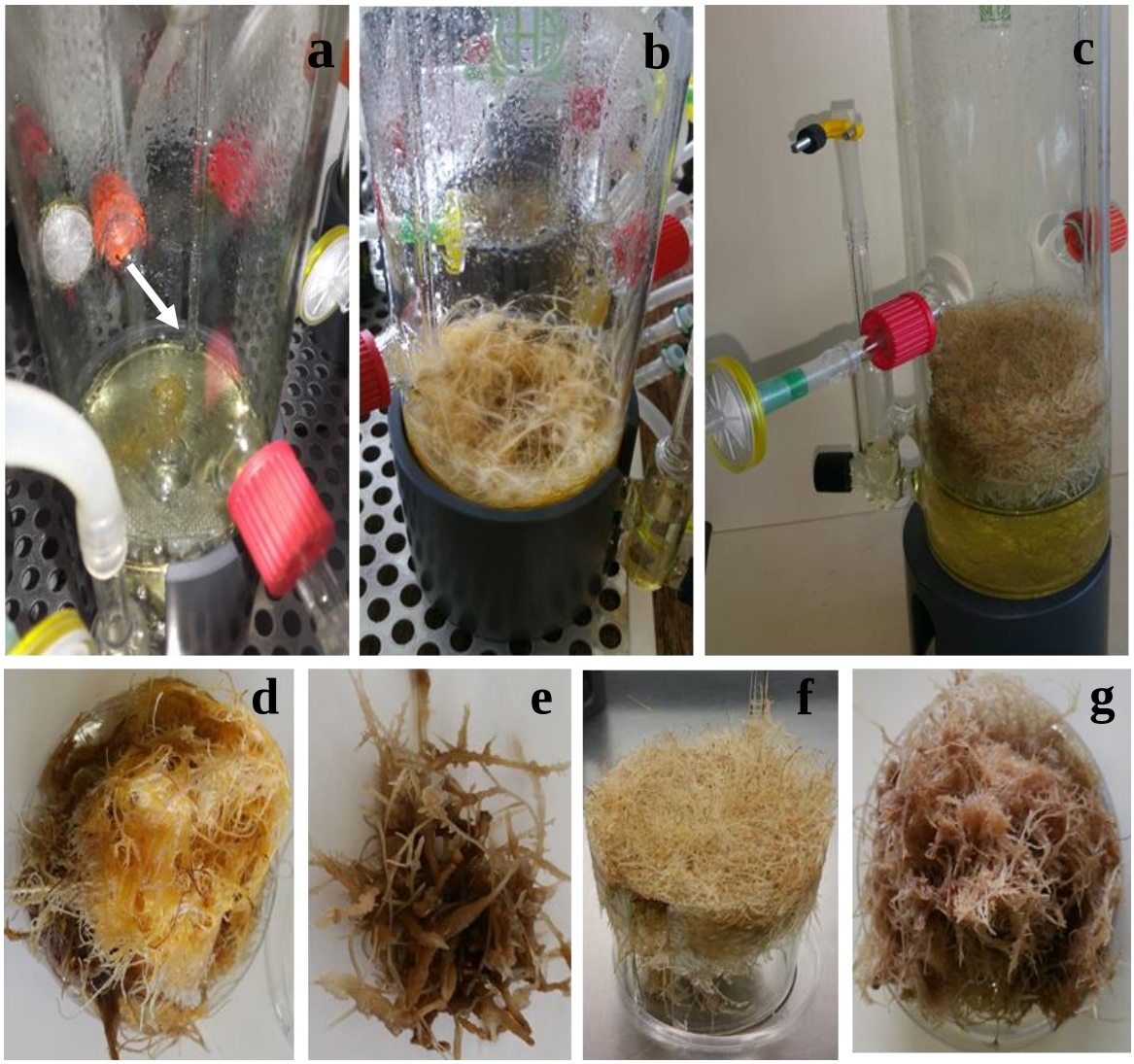

Figure 46: Hairy root initiation in a bioreactor, a. Initial inoculum, b,c. Hairy root biomass after 30 days, d.e.f.g. Different Hairy root morphology from different varieties of *Hyoscyamus niger* and *Sesamum indicum*.

4.6. Biomedical Activity

4.6.1. *Caenorhabditis elegans* Mortality and LC_{90}, LC_{50} assessment

The in *vitro* antinematodal activity of the crude extract, fixed oil, and essential oils from Henbane and Sesame root, seed, and hairy root culture against *C. elegans* were performed represented as percentage mortality of nematode and lethal concentrations LC_{50} and LC_{90}.

4.6.1.1. Essential oils

Essential oils extracted from 3 plant variates of *H. niger*, revealed yields that ranged from <0.05% to 0.1% (v/w). Essential oils were tested for the activity against *Caenorhabditis elegans* via direct contact bioassays. The assays performed with deionized water which showed an average mortality of (3%), which was considered as natural mortality. The mortality due to methanol used as the EO solvent can be considered negligible since 3% of the solvent was used (Guo *et al.*, 2018) (Figure 47).

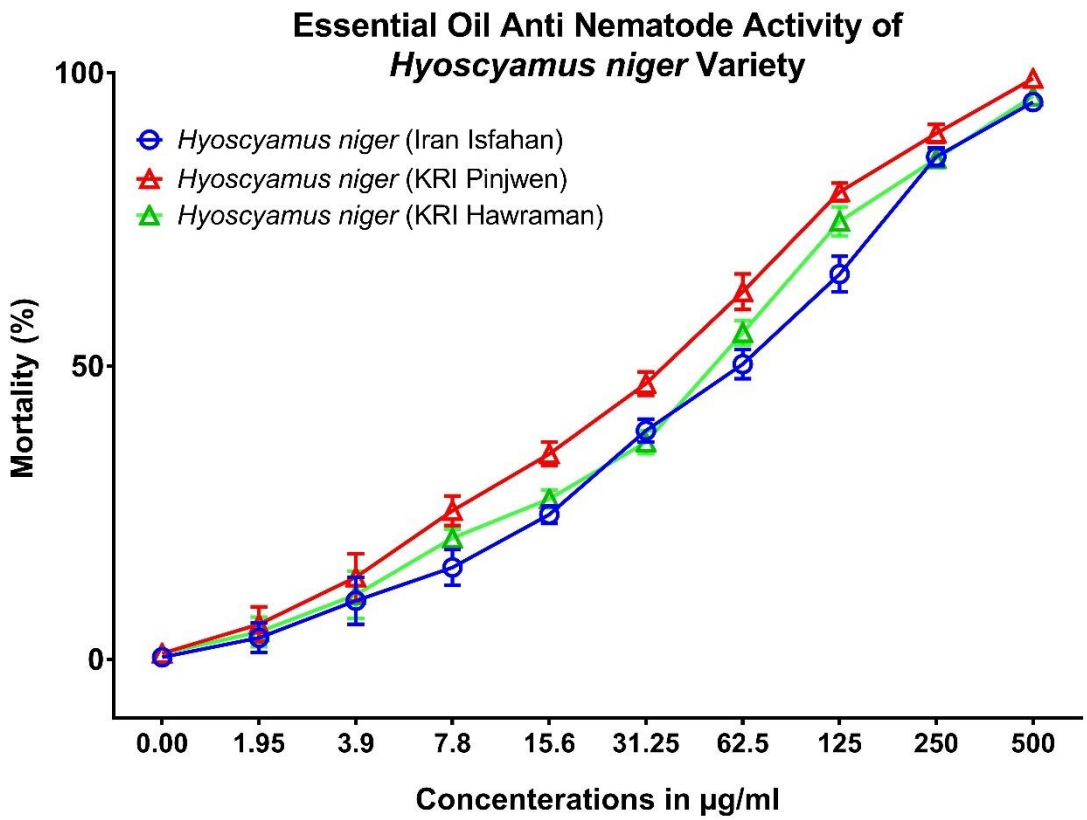

Figure 47: Mortality percentages caused by essential oil extracted from KRI Pinjwen, KRI Hawraman, and Iran Isfahan varieties seeds against *C. elegans*.

The most active EOs, showing the highest mortalities ≤100% at 500 µg/mL, occurred within the varieties Iran Isfahan, KRI Pinjwen, and KRI Hawraman. Within varieties, KRI Pinjwen, with ≤99% highest mortality, was achieved. The varieties (Iran Isfahan and KRI Hawraman) EOs mortalities were ≤95% and ≤96%, respectively. The different results recorded with the same species returned to the diversity of the secondary metabolite. In this manner, the chemotype of the nematotoxic bearing plant species should be taken into consideration when choosing a nematotoxic EO bearing-species. Highest *C. elegans* mortality (99%) was also observed with black henbane EOs (Table 9).

At all, the concentrations tested 39.1 µg/mL KRI Pinjwen, EOs revealed to be the most active. The lethal doses (LC_{50} & LC_{90}) of these EOs were calculated since the *C. elegans* can proliferate even from a very small population because they have a high reproductive capability and. LC_{50} ranged from 39.1, 48.2, and 58.1 µg/mL, while LC_{90} ranged from 247.3, 318.5, and 356.6 µg/mL for the varieties KRI Pinjwen, KRI Hawraman, and Iran Isfahan respectively (Figure 48) and (Table 8).

Table 8: LC values in (µg/mL) for *C. elegans* mortality caused by essential oil of *H*yoscyamus *niger*.

Plant varietiesy Essential Oil	**LC_{50}**	**LC_{90}**
Hyoscyamus niger (KRI Pinjwen)	39.1	268.5
Hyoscyamus niger (KRI Hawraman)	48.2	318.5
Hyoscyamus niger (Iran Isfahan)	58.1	356.6

Table 9: Mortality % anti-nematode activity of *Hyoscyamus niger* essential oil against *C. elegans*.

Concentrations µg/mL	*Hyoscyamus niger*		
	Mortality % ± SD		
	Hawraman	Pinjwen	Iran Isfahan
1.95	4.6±2.5	6.0±3.0	3.6±2.5
3.9	11.0±4.0	14.0±4.0	10.0±4.0
7.8	20.6±1.5	25.3±2.5	15.6±3.0
15.6	27.3±1.5	35.0±2.0	24.6±1.5
31.25	37.0±2.0	47.0±2.0	39.0±2.0
62.5	55.6±2.0	51.6±1.5	50.3±2.5
125	74.6±2.5	79.6±1.5	65.6±3.0
250	85.3±1.5	89.6±1.5	85.6±1.5
500	96.0±1.0	99.0±1.0	95.0±1.0

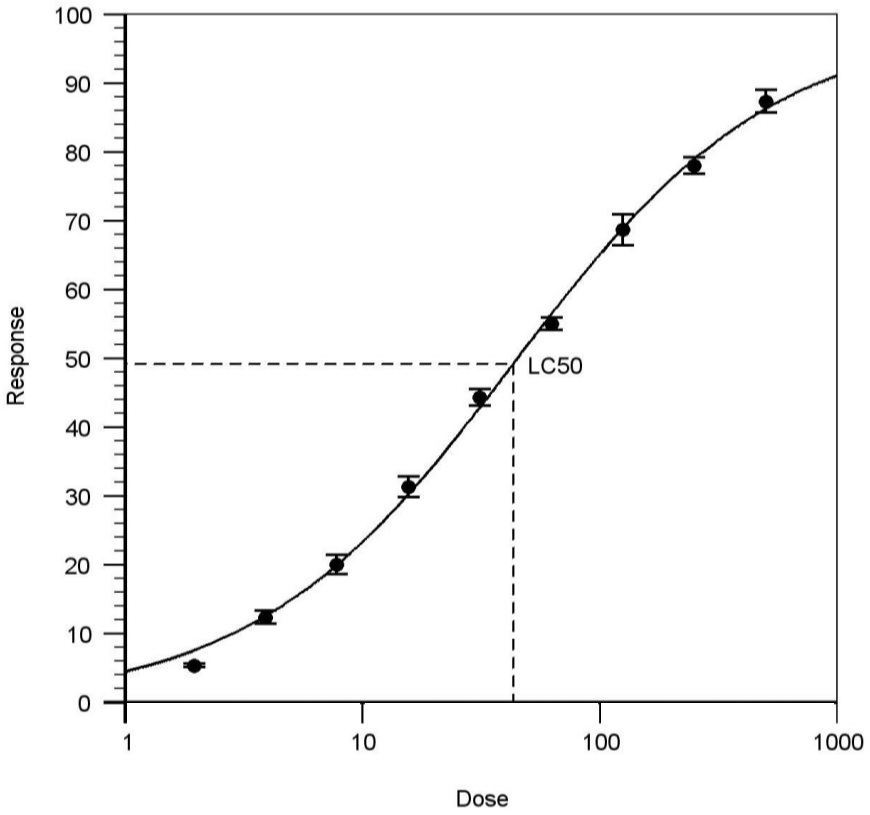

Figure 48: Lethal concentration LC_{50} of Henbane (KRI Hawraman) varieties essential oil on *C. elegans lethality*, after incubation, the percentage of dead nematodes was determined by counting the number of viable and dead worms in each well based on movement.

4.6.1.2. Hairy root extracts

The results showed that using the liquid culture method for testing *C. elegans* mortality is a fast and reliable way to propagate and conduct the lethality test. This method can be used to screen plant secondary metabolite extracts for their potential as an antihelmintic activity. The method is slightly modified from previously described tests (Stiernagle and Maintenance, 2006).

Reviewing the available literature, no evidence was available regarding the anthelmintic activity of hairy root culture extract of *Hyoscyamus niger*. The hairy root extraction was used with concentrations of 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500 µg/mL were used during this study. The results showed that all the extracts exhibit anthelmintic activity by causing death to *C. elegans* nematodes during a period of 48 hours (Figures 49 and 50).

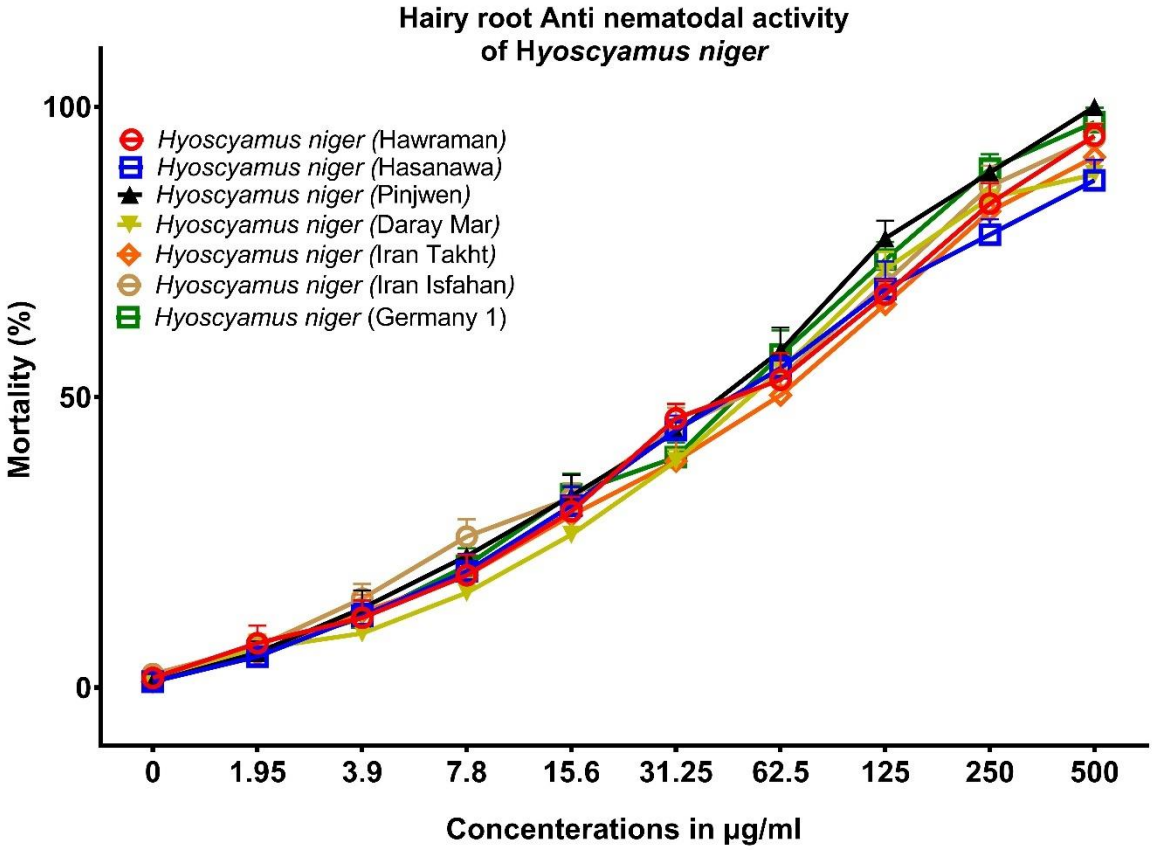

Figure 49: Mortality percentages caused by hairy root methanolic extracted from *Hyoscyamus niger* varieties against *C. elegans*.

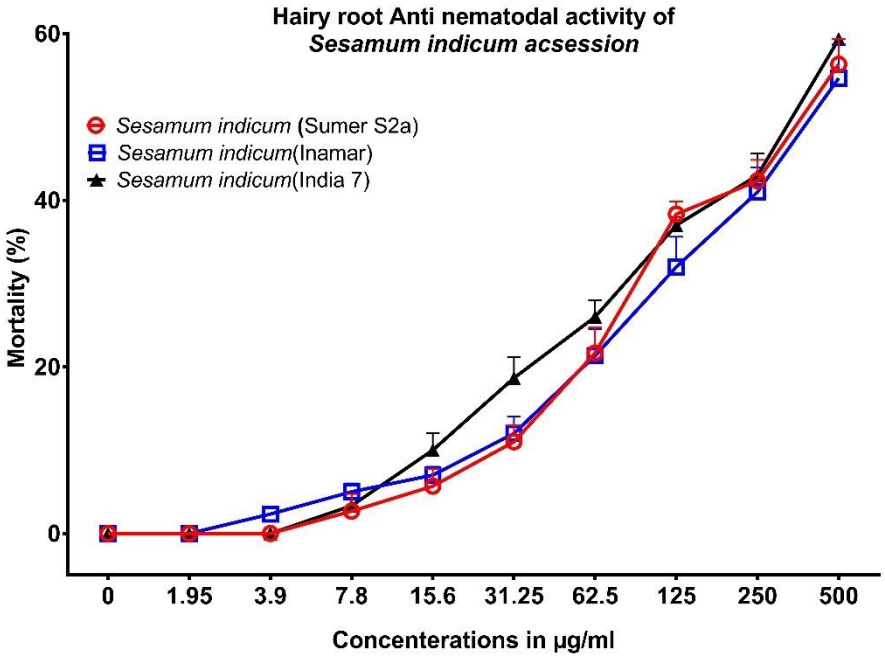

Figure 50: Mortality percentages caused by hairy root methanolic extracted from *Sesamum indicum* varieties against *C. elegans*.

The concentrations of 500 µg/mL methanol extract from *H. niger* varieties exhibit *C. elegans* % mortality of 100 , 94.6 and 97.3%, for KRI Pinjwenand, Iran Isfahan, and Germany 1 respectively, furthermore, all other henbane varieties show significant mortality in *C. elegans* (Table 10), while for *S. indicum* showed positive mortality 56.3, 54.6, and 59.3%, for Sumer S2a, Inamar, and India 7 varieties respectively (Table 11). *S. indicum* extract showed moderate inhibitory effect comparing with *H. niger*.

Table 10: Mortality % anti-nematode activity of *Hyoscyamus niger* hairy root against *C. elegans*.

Concentrations µg/mL	*Hyoscyamus niger*						
	Mortality % ± SD						
	Hawraman	Hasanawa	Pinjwen	Daray Mar	Iran Takht	Iran Isfahan	Germany 1
1.95	7.6±3.0	5.3±0.5	6.0±2.0	6.6±2.5	5.6±1.5	7.0±2.0	6.3±1.5
3.9	12.0±3.0	12.3±2.0	13.6±3.0	9.3±3.0	13.0±3.0	15.3±2.5	12.0±2.0
7.8	19.3±3.5	20.0±3.0	22.6±1.5	16.3±2.5	19.3±2.5	26.0±3.0	21.0±3.0
15.6	30.3±2.5	31.3±3.2	33.0±3.6	26.3±4.0	29.6±3.0	32.6±2.5	33.3±3.5
31.2	46.3±2.5	44.3±2.5	44.0±1.0	39.0±2.0	39.0±5.5	44.6±3.5	39.6±2.5
62.5	53.0±4.5	55.0±2.0	58.0±4.0	54.6±2.5	50.3±3.0	53.3±2.5	57.3±4.1
125	67.6±2.5	68.6±4.7	77.3±3.0	72.0±3.0	66.0±3.6	69.6±2.5	73.6±3.0
250	83.3±3.5	78.0±2.6	88.6±1.5	84.3±2.5	82.0±5.5	86.3±3.5	89.3±2.5
500	95.0±2.0	87.3±3.0	100±00	88.3±2.5	92.3±1.5	94.6±2.5	97.3±2.5

Table 11: Mortality % anti-nematode activity of *Sesamum indicum* hairy root methanolic extracts against *C. elegans*.

Concentration µg/mL	*Sesamum indicum*		
	Mortality % ± SD		
	Sumer S2a	Inamar	India 7
1.95	0.00	0.00	0.00
3.9	0.00	2.33±0.5	0.00
7.8	2.67±2.0	5.00±1.0	3.33±1.5
15.6	5.67±2.0	5.67±1.0	10.00±2.0
31.25	11.00±2.0	12.00±2.0	18.67±2.5
62.5	21.67±3.0	21.33±3.2	26.00±2.0
125	38.33±1.5	32.00±3.6	37.00±1.0
250	42.33±2.5	41.00±3.0	43.00±2.6
500	56.33±3.0	54.67±3.4	59.33±1.5

The lethal doses (LC_{50} & LC_{90}) of the hairy root methanol extract were calculated. *Hyoscyamus niger* ethanol extract LC_{50} ranged from 48.2, 44.9, 39.7, 48.4, 57.8, 44.7, and 43.6 µg/mL, while the LC_{90} ranged from 377.0, 665.7, 262.6, 505.8, 439.7, 355.1, and 289.6 µg/mL for the varieties KRI Hawraman, KRI Hasanawa, KRI Pinjwen, KRI Daray Mar, Iran Takhte, Iran Isfahan, and Germany 1 respectively (Table 12) and (Figure 51).

The LC_{90} ranged from 299.4, 387.1, and 323.7 µg/mL for the varieties India 7, Inamar, and Sumer S2a, respectively, while the methanol extract LC_{50} of *S. indicum* was very high and considered as (undefined) (Table 12).

Table 12: LC values in (µg/mL) for *C. elegans* mortality caused by hairy root of *Hyoscyamus niger* and *Sesamum indicum*.

Plant varieties	LC_{50} (µg/mL)	LC_{90} (µg/mL)
Hyoscyamus niger (KRI Hawraman)	48.2	377.0
Hyoscyamus niger (KRI Hasanawa)	44.9	665.7
Hyoscyamus niger (KRI Pinjwen)	39.7	262.6
Hyoscyamus niger (KRI Daray Mar)	48.4	505.8
Hyoscyamus niger (Iran Takhte)	57.8	439.7
Hyoscyamus niger (Iran Isfahan)	44.7	355.1
Hyoscyamus niger (Germany 1)	43.6	289.6
Seamum indicum(India 7)	299.4	Undefined
Seamum indicum(Inamar)	387.1	Undefined
Seamum indicum(Sumer S2a)	323.7	Undefined

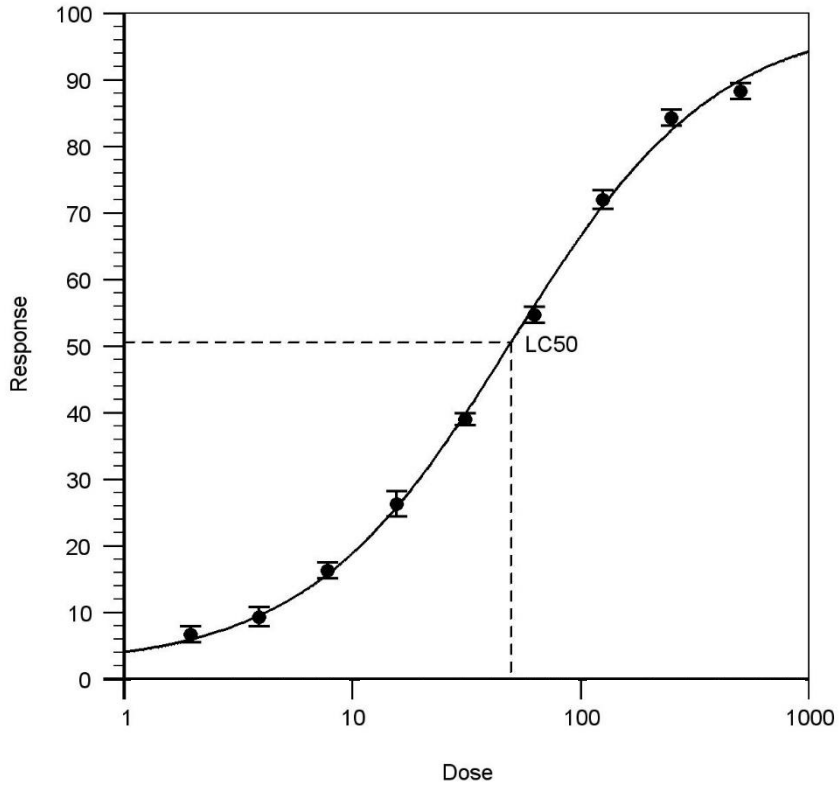

Figure 51: Lethal concentration LC_{50} of Henbane (Darai Mar) varieties essential oil on *C. elegans* lethality, after incubation, the percentage of dead nematodes was determined by counting the number of viable and dead worms in each well based on movement.

4.6.1.3. Root extract

This study was performed on the *H. niger* root extract for anthelmintic activity. the results showed positive mortality of the henbane root methanol extract. The results indicate that a fast and reliable way to propagate and perform the lethality test is to examine the biomedical activity of plants against *C. elegans* through the liquid cultivation method. This method can be used to screen for possible anthelmintic activity secondary plant metabolite extracts and compounds (Stiernagle and Maintenance, 2006).

Nine serial concentrations of 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500 µg/mL of methanolic extract in both plants were used to conduct this study. The results showed that all the extracts, tested at all the doses, caused anthelmintic activity by causing death to *C.elegans* nematodes during a period of 48 hours (Figures 52 and 53).

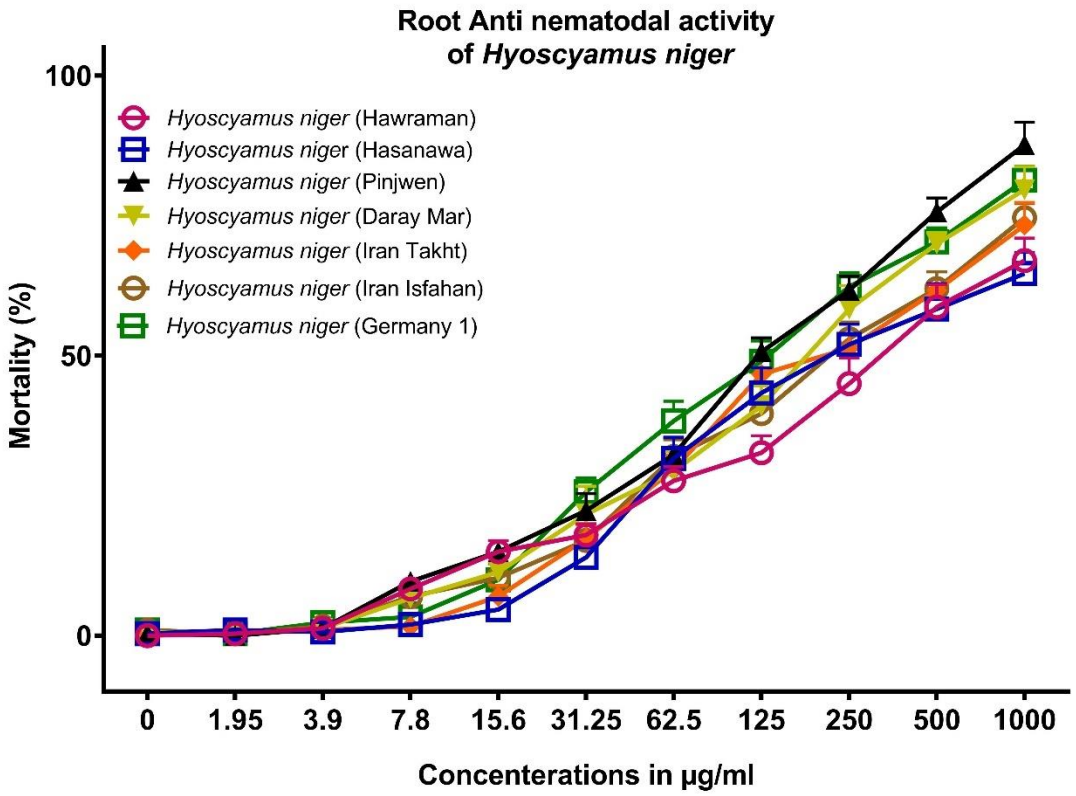

Figure 52: Mortality percentages caused by root methanolic extract from *Hyoscyamus niger* varieties against *C. elegans.*

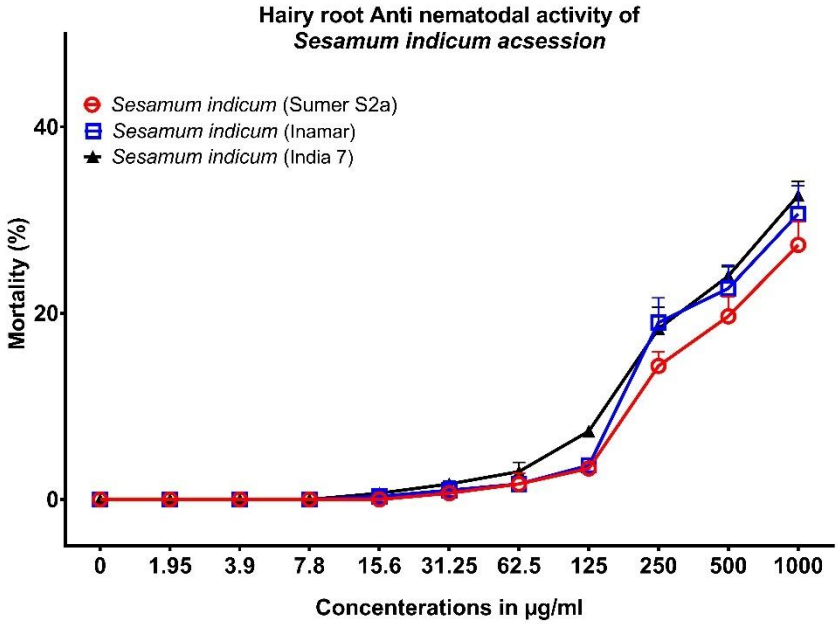

Figure 53: Mortality percentages caused by root extract from *Sesamum indicum* varieties against *C. elegans*.

The concentrations of 1000 µg/mL of ethanol extract recorded 87.7, 81.3, and 79.7% in *C. elegans* mortality for the *Hyoscyamus niger* varieties KRI Pinjwen, Germany 1, and KRI Daray Mar. Furthermore, all other henbane varieties showed significant mortality against *C. elegans* mortality (Table 13). While *S. indicum* exhibited positive mortality only for 1000 µg/mL (Table 14).

Table 13: Mortality % anti-nematode activity of *Hyoscyamus niger* root extract against *C. elegans*.

Concentrations µg/mL	*Hyoscyamus niger*						
	Mortality % ± SD						
	Hawraman	Hasanawa	Pinjwen	Daray Mar	Iran Takht	Iran Isfahan	Germany 1
1.95	0.3±0.5	0.6±0.5	1.3±0.5	0.3±0.5	0.3±0.5	0.3±0.5	0.3±0.5
3.9	1.3±1.1	1.6±0.5	2.3±0.5	1.3±1.5	1.0±1.0	1.6±1.5	2.3±1.5
7.8	8.3±1.5	3.0±1.0	9.6±1.5	6.6±1.5	1.6±0.5	7.0±2.0	3.3±2.5
15.6	15.0±2.0	4.6±0.5	15.0±2.0	11.3±1.5	7.0±2.0	10.3±2.5	10.0±3.0
31.25	18.0±2.0	14.0±2.0	22.3±3.0	21.6±5.0	17.3±2.5	17.0±3.0	25.6±2.5
62.5	27.6±2.5	31.6±3.7	32.3±3.0	29.3±3.5	29.6±2.5	32.0±3.0	38.3±3.5
125	32.6±3.0	43.3±4.5	50.6±2.5	41.0±3.6	46.6±1.5	39.6±2.5	49.0±3.6
250	45.0±4.5	52.0±3.6	61.6±2.5	58.3±4.1	51.3±2.5	53.0±3.0	62.3±2.5
500	58.6±4.0	58.3±4.5	75.6±2.5	70.0±2.0	61.6±1.5	62.0±3.0	70.3±2.5
1000	67.0±4.0	64.6±3.7	87.6±4.0	79.6±4.1	73.3±4.0	74.7±2.5	81.3±2.5

Table 14: Mortality % anti-nematode activity of *Sesamum indicum* root extract against *C. elegans*.

Concentrations µg/mL	*Sesamum indicum*		
	Mortality % ± SD		
	Sumer S2a	Inamar	India 7
1.95	0.0	0.0	0.0
3.9	0.0	0.0	0.0
7.8	0.0	0.0	0.0
15.6	0.0	0.3±0.5	0.6±0.5
31.25	0.6±0.5	1.0±1.0	1.6±0.5
62.5	1.6±1.1	1.6±0.5	3.0±1.0
125	3.3±0.5	3.6±0.5	7.3±0.5
250	14.3±1.5	19.0±2.6	18.3±2.3
500	19.6±2.0	22.6±2.5	24.0±1.0
1000	27.3±2.5	30.6±3.0	32.6±1.5

Sesamum indicum extract did not show any significant inhibitory effect comparing with henbane. *Hyoscyamus niger* methanol extract LC_{50} ranged 319.5, 183.8, 122.6, 175.3, 183.3, 210.3, and 129.5 µg/mL, while the LC_{90} ranged from 12443.3, 1298.8, 1268.5, 3239.3, 2301.7, 3089.9, and 1604.9 µg/mL for the varieties KRI Hawraman, KRI Hasanawa, KRI Pinjwen, KRI Daray Mar, Iran Takhte, Iran Isfahan, and Germany 1 respectively (Table 15) and (Figure 54). The lethal concentration of *Sesamum indicum* root methanol extract was considered as (undefined)

Table 15: LC values in (µg/mL) for *C. elegans* mortality caused by root extract of *Hyoscyamus niger*.

Plant varieties	LC_{50}	LC_{90}
Hyoscyamus niger (KRI Hawraman)	319.5	1244.3
Hyoscyamus niger (KRI Hasanawa)	183.8	1298.8
Hyoscyamus niger (KRI Pinjwen)	122.6	1268.5
Hyoscyamus niger (KRI Daray Mar)	175.3	3239.3
Hyoscyamus niger (Iran Takhte)	183.3	2301.7
Hyoscyamus niger (Iran Isfahan)	210.3	3089.9
Hyoscyamus niger (Germany 1)	129.5	1604.9

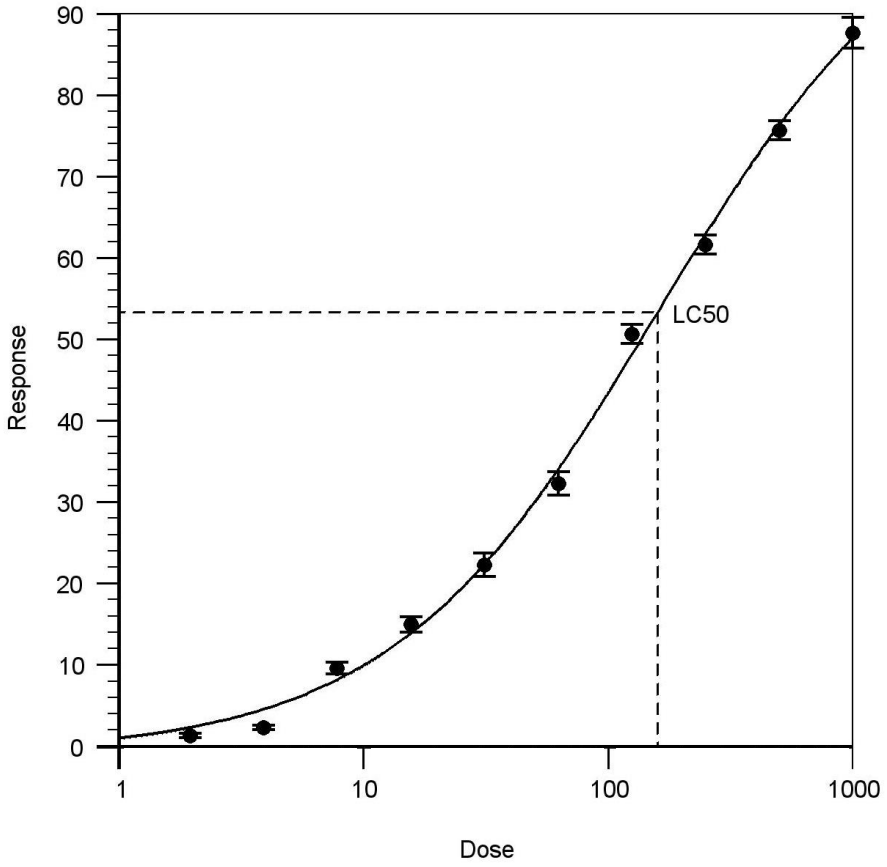

Figure 54: Lethal concentration LC_{50} of Henbane (Pinjwen) varieties essential oil on *C. elegans* lethality, after incubation, the percentage of dead nematodes was determined by counting the number of viable and dead worms in each well based on movement.

4.6.1.4. Seed extract

4.6.1.4.1. Crude extract

The seed was examined for their anthelmintic activity against the *C. elegans* nematode, using different concentrations of crude methanol extracts of 7 varieties of Henbane and 3 varieties of Sesame. The anthelmintic activity of nine concentrations of 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500 µg/mL, was used to conduct this study. The results showed that all the extracts tested at all the concentrations caused anthelmintic activity by causing death to *C.elegans* nematodes after 48 hours (Figures 55 and 56).

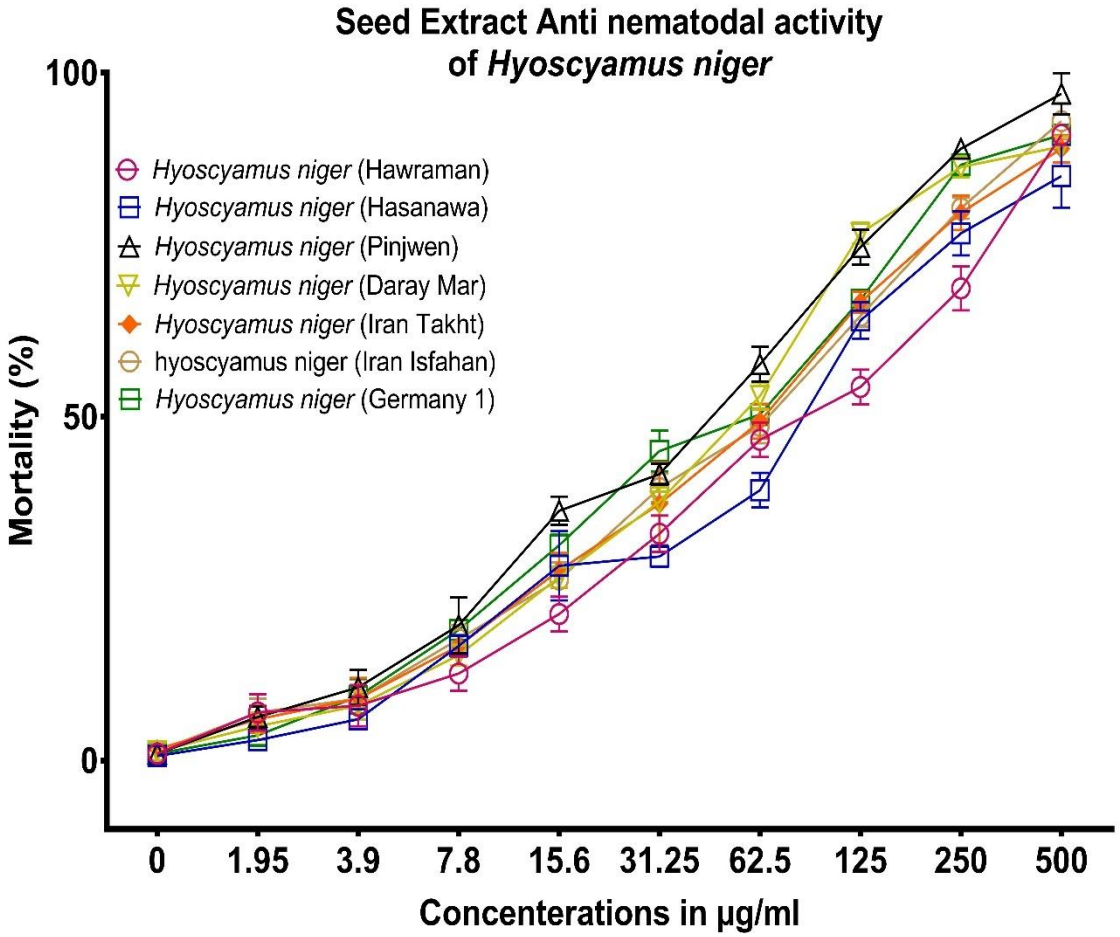

Figure 55: Mortality percentages caused by seed crude extract from *Hyoscyamus niger* varieties against *C. elegans*.

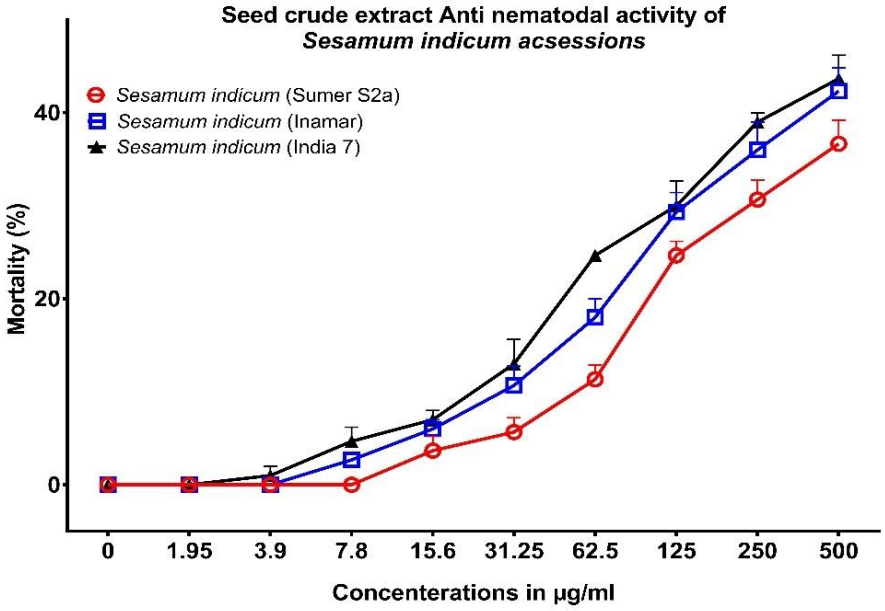

Figure 56: Mortality percentages caused by seed crude extract from *Sesamum indicum* varieties against *C. elegans.*

The seed crude extract at the dose of 500 µg/mL showed the highest mortality 97% for KRI Pinjwen varieties, while the lowest mortality was 85% for KRI Hasanawa varieties, furthermore, all other henbane varieties exhibited mortality against *C. elegans* (Table 16), furthermore *Sesamum indicum* displayed the highest positive mortality of 43.7%, 42.3 and 36.7% for Indian 7, Inamar, and Sumer S2a varieties respectively at 500 µg/mL (Table 17).

Table 16: Mortality % anti-nematode activity of *Hyoscyamus niger* seeds crude extract against *C. elegans*.

Concentrations µg/mL	*Hyoscyamus niger*						
	Mortality % ± SD						
	Hawraman	Hasanawa	Pinjwen	Daray Mar	Iran Takht	Iran Isfahan	Germany 1
1.95	7.0±2.6	3.0±1.0	6.3±1.5	5.0±2.0	6.0±2.0	7.0±2.0	3.6±1.5
3.9	8.0±3.0	6.0±1.0	10.6±2.5	8.0±2.0	9.0±3.0	9.0±3.0	9.3±2.5
7.8	12.7±2.5	16.6±1.5	19.6±4.0	15.3±1.5	16.6±1.5	17.6±2.0	19.0±1.0
15.6	21.3±2.5	28.3±5.0	36.3±2.0	26.6±1.5	27.6±2.5	26.3±2.5	31.3±1.5
31.2	33.0±2.6	29.6±1.5	41.6±1.5	37.6±2.0	37.3±3.0	39.6±2.0	45.0±3.0
62.5	46.6±2.5	39.3±2.5	57.6±2.5	53.0±2.0	49.3±2.5	48.6±2.5	50.3±1.5
125	54.3±2.5	64.0±2.6	74.6±2.5	76.6±1.5	66.6±1.5	64.6±1.5	67.0±1.0
250	68.6±3.2	76.6±3.2	89.0±1.0	86.3±1.5	79.6±2.5	80.3±1.5	86.6±1.5
500	91.0±1.0	85.0±4.5	97.0±3.0	89.3±1.1	89.0±2.0	93.0±2.6	91.0±1.0

Table 17: Mortality % anti-nematode activity of *Sesamum indicum* seeds crude extract against *C. elegans.*

Concentrations µg/mL	***Sesamum indicum***		
	Mortality % ± SD		
	Sumer S2a	**Inamar**	**India 7**
1.95	0.00	0.00	0.00
3.9	0.00	0.00	1.0±1.0
7.8	0.00	2.6±0.5	4.6±1.5
15.6	3.6±1.5	6.0±1.0	7.0±1.0
31.25	5.6±1.5	10.6±2.0	13.0±2.6
62.5	11.3±1.5	18.0±2.0	24.6±0.5
125	24.6±1.5	29.3±2.0	30.0±2.6
250	30.6±2.0	36.0±3.0	39.0±1.0
500	36.6±2.5	42.3±2.5	43.6±2.5

The inhibitory activity of both plant verities was studied. *Sesamum indicum* seed crude extract did not show a significant lethal effect at lower concentrations, but significant results were recorded when used at a high concentration of 500 µg/mL.

Hyoscyamus niger seed crude extract showed LC_{50} ranged from 75.4, 76.9, 41.0, 47.9, 68.4, 69.9, and 58.6 µg/mL, while the LC_{90} ranged from 525.1, 623.8, 290.0, 408.6, 529.3, 627.8, and 419.9 µg/mL for varieties KRI Hawraman, KRI Hasanawa, KRI Pinjwen, KRI Daray Mar, Iran Takhte, Iran Isfahan, and Germany 1 respectively, (Table 18) and (Figure 57). The lethal concentration of *Sesamum indicum S. indicum* seed crude extract was considered as (undefined)

Table 18: LC values in (µg/mL) for *C. elegans* mortality caused by seeds crude extract of *Hyoscyamus niger*.

Plant varieties	LC_{50} µg/mL	LC_{90} µg/mL
Hyoscyamus niger (KRI Hawraman)	75.4	525.1
Hyoscyamus niger (KRI Hasanawa)	76.9	623.8
Hyoscyamus niger (KRI Pinjwen)	41.0	290.0
Hyoscyamus niger (KRI Daray Mar)	47.9	408.6
Hyoscyamus niger (Iran Takhte)	68.4	529.3
Hyoscyamus niger (Iran Isfahan)	69.9	627.8
Hyoscyamus niger (Germany 1)	58.6	419.9

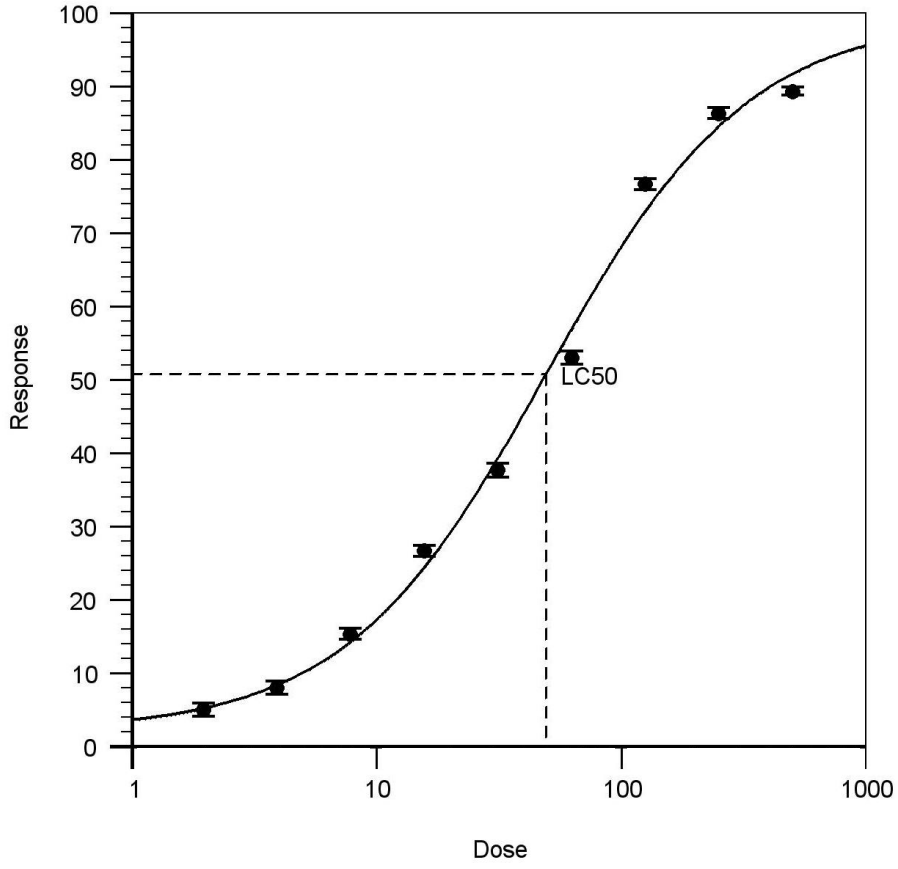

Figure 57: Lethal concentration LC_{50} of Henbane (KRI Hasanawa) variety seed crude extract on *C. elegans* lethality, after incubation, the percentage of dead nematodes was determined by counting the number of viable and dead worms in each well based on movement.

4.6.1.4.2. Fixed oil extract

The fixed oil of the dry seeds *of H. niger* and *S. indicum* was extracted using a modified Soxhlet apparatus. The anthelmintic activity of nine oil extract concentrations of 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500 µg/mL were used during this study. The results showed that all the extracts tested caused anthelmintic activity by causing death to *C.elegans* nematodes up to a period of 48 hours, as shown in (Figures 58 and 59).

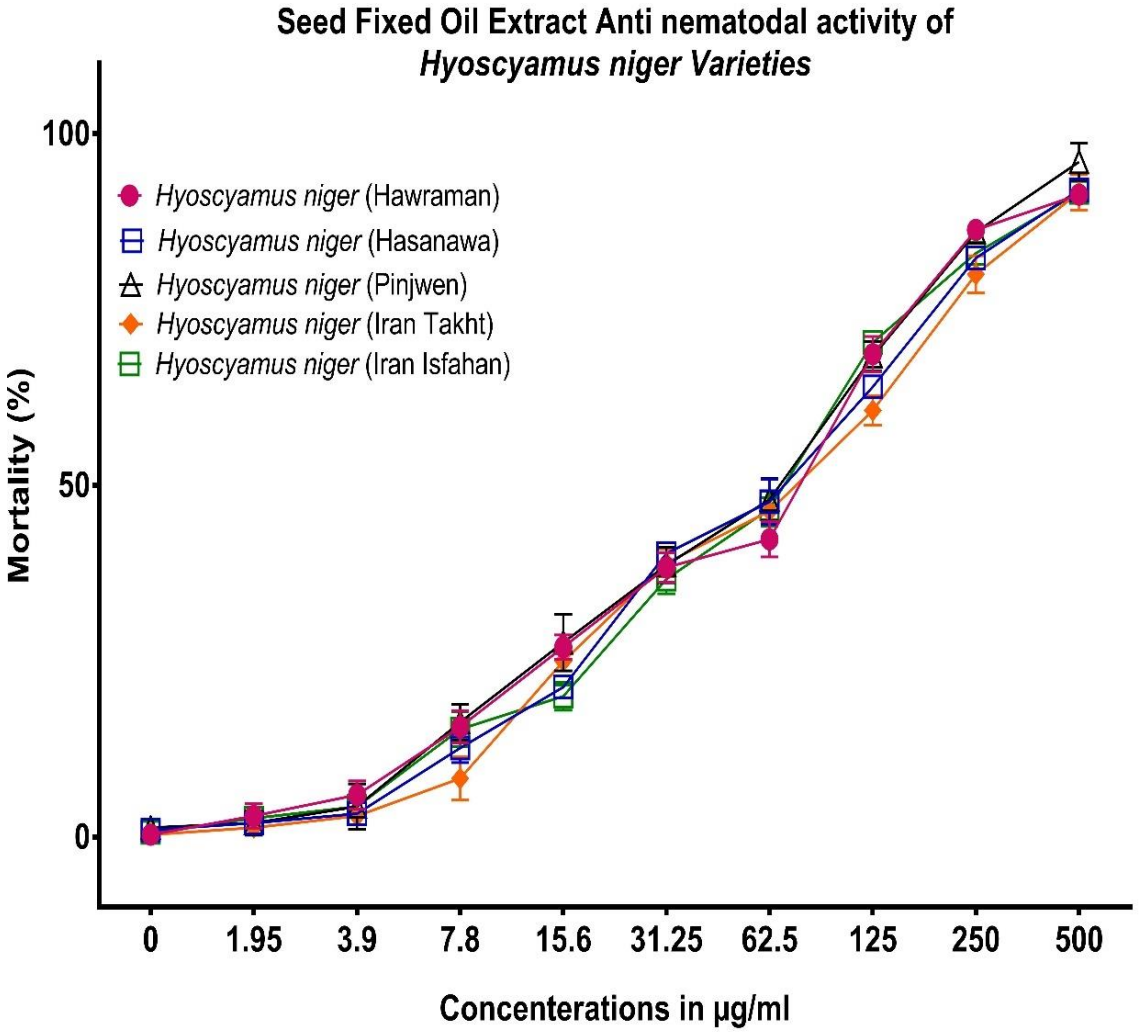

Figure 58: Mortality percentages caused by seed fixed oil extract from *Hyoscyamus niger* varieties against *C. elegans*.

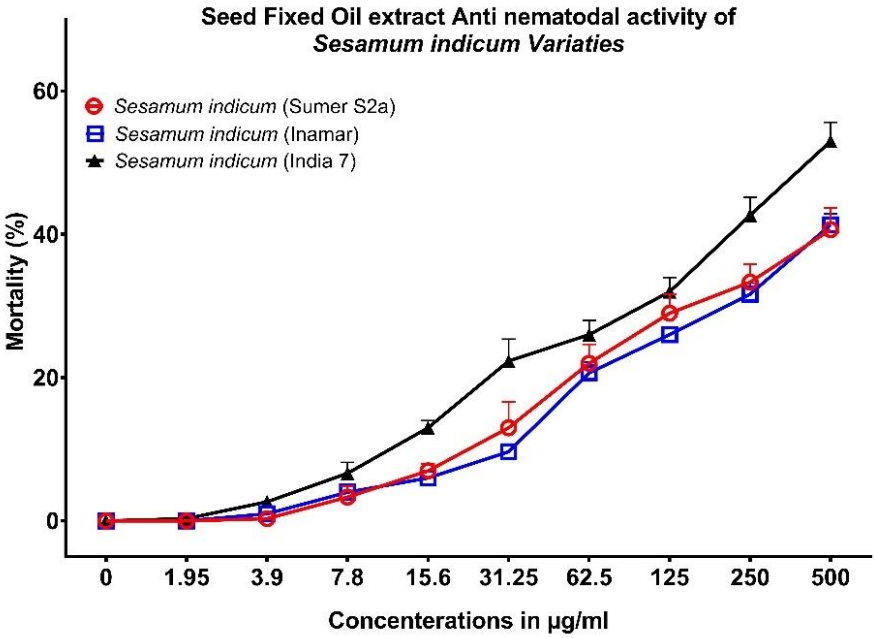

Figure 59: Mortality percentages caused by seed fixed oil extract from *Sesamum indicum* varieties against *C. elegans*.

Nine doses of fixed oil from the seed of *H. niger* and *S. indicum* were examined for their anthelmintic activity. The dose, 500 µg/mL, showed a significant mortality of 96% for KRI Pinjwen varieties. The lowest mortality was 91.33% for KRI Hawraman varieties at 500 µg/mL. Furthermore, all other henbane varieties shoed significant mortality (Table 19), furthermore *Sesamum indicum* showed the highest positive mortality was 53% for Indian 7 varieties at 500 µg/mL (Table 20).

Table 19: Mortality % anti-nematode activity of *Hyoscyamus niger* seed fixed oil against *C. elegans*.

Concentrations	*Hyoscyamus niger*				
	Mortality (%)				
	Hawraman	**Hasanawa**	**Pinjwen**	**Iran Takht**	**Iran Isfahan**
1.95	3.0±1.7	2.0±0.0	20±1.7	1.3±0.5	2.6±0.5
3.9	6.0±2.0	3.3±1.5	4.3±3.2	3.0±1.0	4.3±0.5
7.8	15.6±2.3	12.6±2.0	16.3±2.5	8.3±3.0	5.3±2.5
15.6	21.0±1.7	15.3±1.5	21.6±4.0	19.0±1.0	14.0±2.0
31.25	31.3±2.0	33.3±1.5	31.6±2.5	32.0±2.0	29.6±2.0
62.5	42.3±2.5	47.6±3.2	48.0±3.0	46.3±1,5	46.6±2.5
125	68.6±2.5	64.0±1.0	68.3±2.0	60.6±2.0	70.3±1.5
250	86.3±1.5	82.3±1.5	86.0±1.0	80.0±2.6	83.0±1.0
500	91.3±1.5	92.0±1.0	96.0±2.6	91.6±2.5	91.6±0.5

Table 20: Mortality % anti-nematode activity of *Sesamum indicum* seed fixed oil against *C. elegans.*

Concentrations	*Sesamum indicum*		
	Mortality (%)		
	Sumer S2a	**Inamar**	**India 7**
1.95	0.00	0.00	0.3±0.5
3.9	0.3±0.5	1.0±1.0	2.7±0.5
7.8	3.3±1.5	4.0±1.0	6.7±1.5
15.6	7.0±1.0	6.0±1.0	13.0±1.0
31.25	13.0±3.6	9.6±0.5	22.3±3.0
62.5	22.0±2.6	20.6±1.5	26.0±2.0
125	29.0±2.6	26.0±1.0	32.0±2.0
250	33.3±2.5	31.6±1.5	42.7±2.5
500	40.7±3.0	41.3±1.5	53.0±2.6

Sesamum indicum seed oil extract did not show considerable mortality at lower concentrations, but with a high dose showed inhibitory effect.

Hyoscyamus niger seed shows LC_{50} ranged from 60.6, 61.9, 57.0, 66.9, and 60.1 µg/mL, While the LC_{90} ranged from 391.8, 438.9, 339.1, 473.9, and 410.5 µg/mL for the varieties KRI Hawraman, KRI Hasanawa, KRI Pinjwen, Iran Takhte, and Iran Isfahan respectively, (Table 21) and (Figures 60).

The LC_{50} of *S. indicum* Indian 7 variety seed fixed oil extract, were 427.7 µg/mL while the Iran Takhte and Iran Isfahan was very high and considered as undefined while for LC_{90} of *S. indicum* Indian 7 variety was 5706.1 µg/mL but for Iran Takhte and Iran Isfahan was very high and considered as undefined (Table 21).

Table 21: LC values in (µg/mL) for *C. elegans* mortality caused by seed fixed oil of *Hyoscyamus niger* and *Sesamum indicum*.

Plant varieties	LC_{50} µg/mL	LC_{90} µg/mL
Hyoscyamus niger (KRI Hawraman)	67.2	398.8
Hyoscyamus niger (KRI Hasanawa)	69.3	423.9
Hyoscyamus niger (KRI Pinjwen)	66.4	360.8
Hyoscyamus niger (Iran Takhte)	73.7	475.0
Hyoscyamus niger (Iran Isfahan)	68.9	402.3
Seamum indicum(India 7)	427.7	5706.1

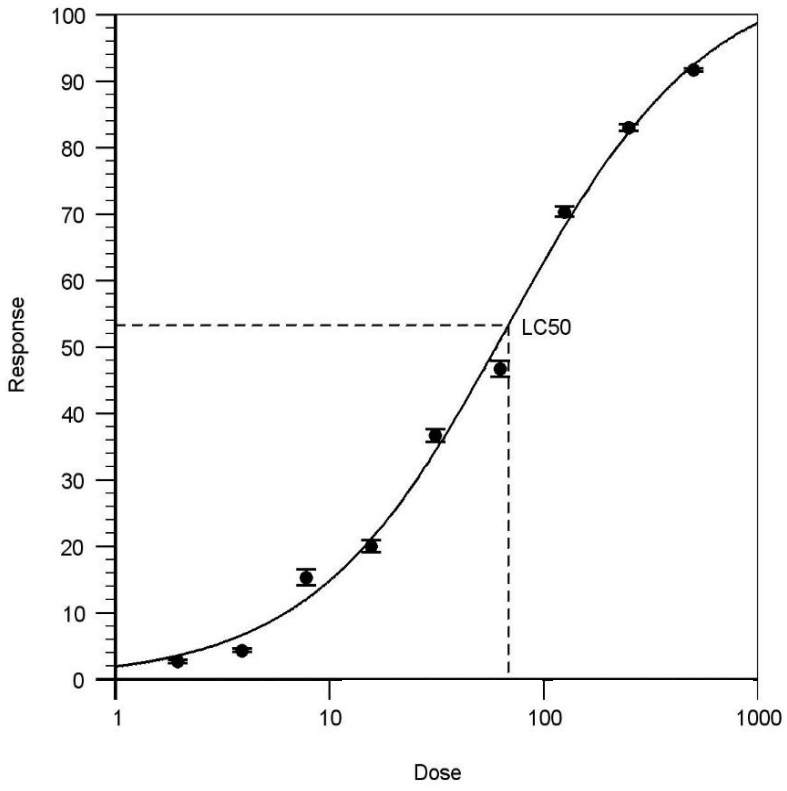

Figure 60: Lethal concentration LC_{50} of Henbane (Iran Takhte) variety seed fixed oil on *C. elegans* lethality, after incubation, the percentage of dead nematodes was determined by counting the number of viable and dead worms in each well based on movement.

4.6.1.5. Thermal Release Anti nematode activity

Plant seed thermal release was exposed to *C. elegans* for one hour at 0, 50, 60, 70, 80, 90, and 100°C for thermal release direct diffusion to evaluate thermo-emission compounds efficiently at various temperatures of the tests compounds on nematode mortality. The performance of the model system and the effects of thermal release compound on test nematodes were established in a series of preliminary trials conducted with the *C. elegans*. In the example provided in (Figure 61) and (Table 22). These results showed that exposure to thermal release from *H.niger* lyophilized seeds was lethal for *C. elegans* while the thermal release from *Sesamum indicum*, *Lens esculenta*, and *Cicer arietinum* plant lyophilized seeds was not significantly lethal to *C. elegans* (Figure 62).

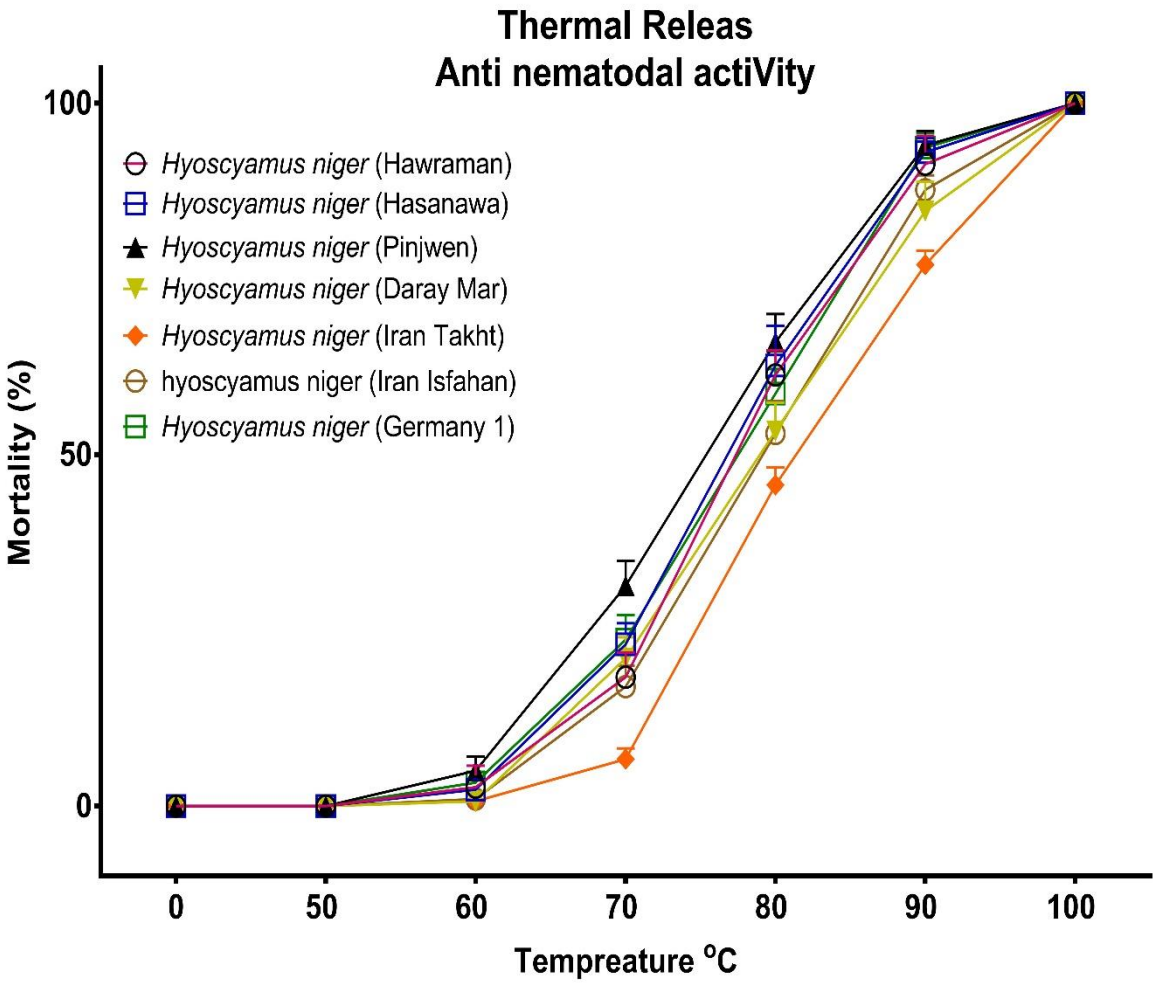

Figure 61: Mortality percentages caused by seed thermal release from *Hyoscyamus niger* varieties against *C. elegans*.

C. elegans mortality with thermal release protocol varied according to the plant varieties and the incubation temperature. The dose of 10 g of lyophilized seeds from *H. niger* plant showed significant maximum mortality of 100% for all varieties at 100^{o}C and 94 % for KRI Pinjwen varieties at 90^{o}C.

While the lowest mortality was at 60°C, all other henbane varieties showed significant mortality to *C. elegans* mortality at 80°C, 90°C and 100°C. *Sesamum indicum*, *Cicer arietinum*, and *Lens esculenta* did not show any positive mortality against *C. elegans* nematode (Figures 63 and 64).

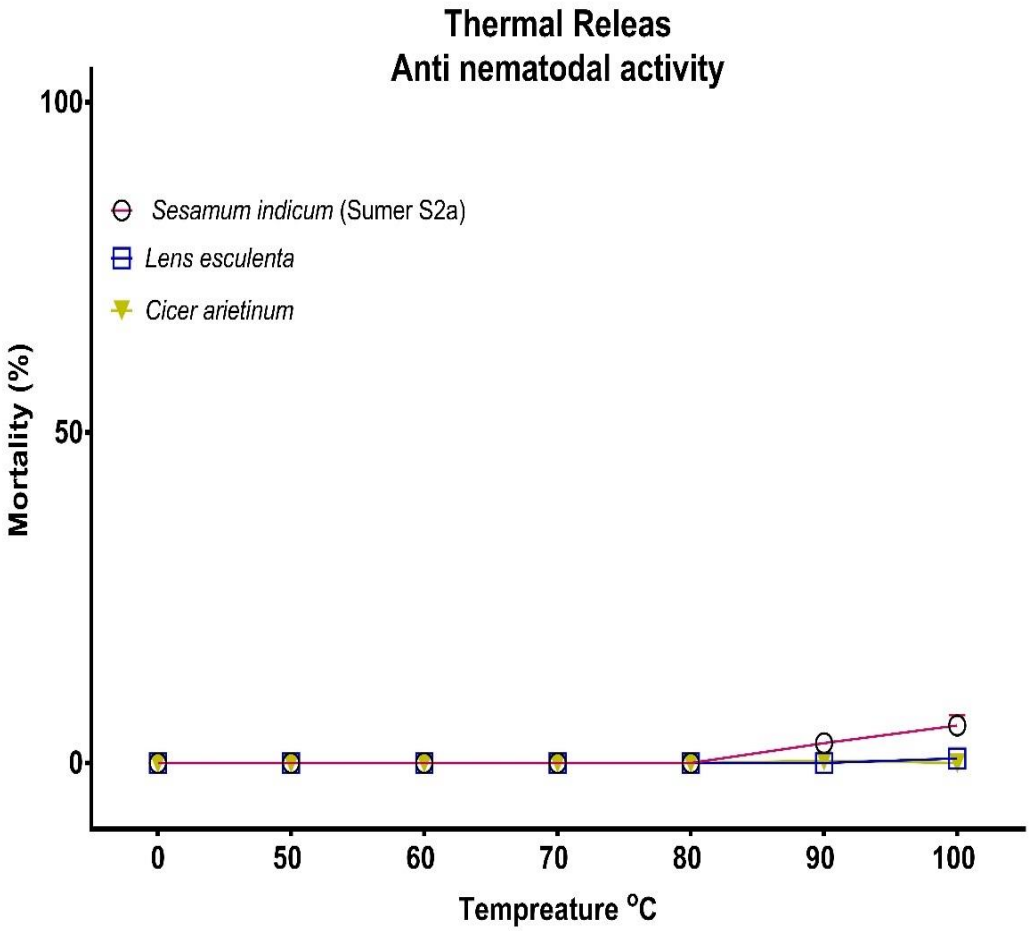

Figure 62: Mortality percentages caused by seed thermal release from *Sesamum indicum, Cicer aritinum,* and *Lens esculenta* varieties against *C. elegans*.

Table 22: Mortality % anti-nematode activity of *Hyoscyamus niger* seed thermal release against *C. elegans*.

Temperatures °C	*Hyoscyamus niger*						
	Mortality % ± SD						
	Hawraman	**Hasanawa**	**Pinjwen**	**Daray Mar**	**Iran Takht**	**Iran Isfahan**	**Germany 1**
0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
50	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
60	2.6±3.0	2.3±1.5	5.0±2.0	0.6±1.1	0.6±1.1	1.0±1.0	3.3±1.5
70	18.3±3.5	23.0±3.0	31.3±3.0	21.0±3.0	6.6±1.5	17.0±3.0	23.6±3.5
80	61.3±3.5	62.6±5.0	66.0±4.0	53.3±4.0	45.6±2.5	53.0±4.5	58.6±3.5
90	91.3±4.0	93.0±2.0	94.0±2.0	84.6±4.1	77.0±2.0	87.6±2.0	93.6±2.0
100	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0	100±0.0

The lethal temperature (LT_{50} & LT_{90}) of thermal release were calculated *Hyoscyamus niger* seeds, thermal release temperature LT_{50} ranged from 77.44, 77.27, 72.86, 80.01, 82.52, 79.79, and 78.11°C, while LT_{90} ranged from 89.49, 88.28, 87.58, 93.32, 94.53, 91.89, and 90.37°C for the varieties KRI Hawraman, KRI Hasanawa, KRI Pinjwen, KRI Daray Mar, Iran Takhte, Iran Isfahan, and Germany 1 respectively (Table 23) and (Figures 63 and 64).

Table 23: LC values in (µg/mL) for *C. elegans* mortality caused by seed thermal release of *Hyoscyamus niger* and *Sesamum indicum*.

Plant varieties	LT_{50} °C	LT_{90} °C
Hyoscyamus niger (KRI Hawraman)	77.44	89.49
Hyoscyamus niger (KRI Hasanawa)	77.27	88.28
Hyoscyamus niger (KRI Pinjwen)	72.86	87.58
Hyoscyamus niger (KRI Daray Mar)	80.01	93.32
Hyoscyamus niger (Iran Takhte)	82.52	94.53
Hyoscyamus niger (Iran Isfahan)	79.79	91.89
Hyoscyamus niger (Germany 1)	78.11	90.37

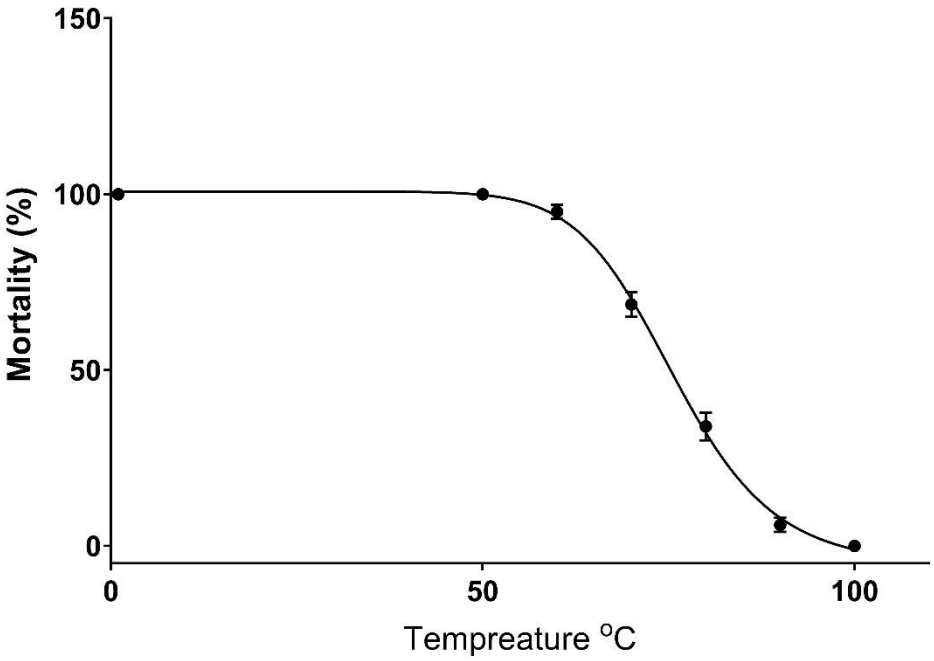

Figure 63: Lethal temperature LT_{50} of Henbane (Pinjwen) varieties thermal release on *C. elegans* mortality, after incubation, the percentage of dead nematodes was determined by counting the number of viable and dead worms in each well based on movement.

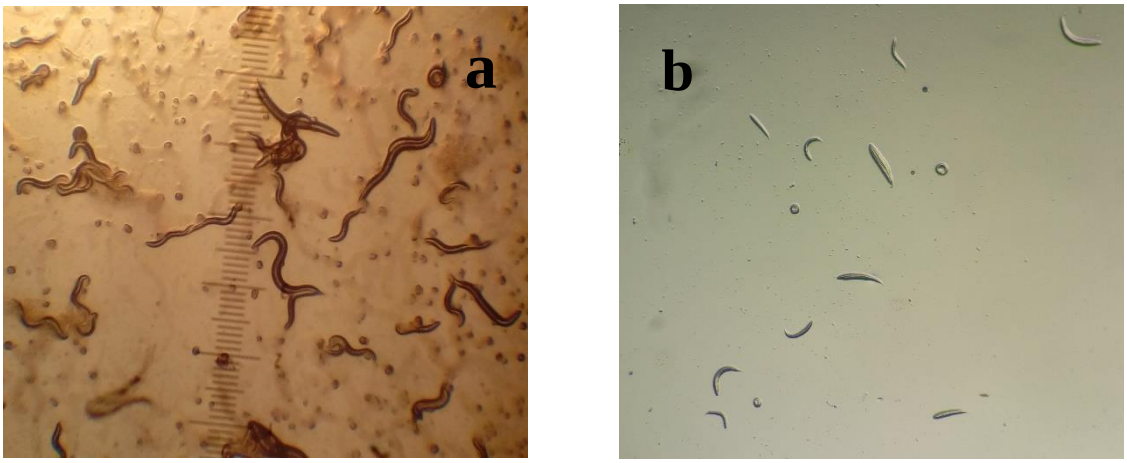

Figure 64: *C. elegans* mortality test results of *Hyoscyamus niger* seed thermal release, a live *C. elegans* before the test. b died *C. elegans* after exposed to seed thermal release.

4.7. Secondary metabolite Profiling

4.7.1. Exudates of hairy roots of *Hyoscyamus niger* and *Sesamum indicum* grew in a Bioreactors and in Flasks

Hairy root cultures of *H. niger* and *S. indicum* were grown in both bioreactors operated in a bubble mode and in traditional flask cultures. The chemical identities of components of the exudates were unknown, preventing quantification with the help of genuine standards. Therefore, an ELSD detector was used, which allows for approximate comparison of concentrations without the use of authentic standards because calibration curves for different compounds are similar (Li *et al.*, 2019). An example of ELSD chromatograms of hairy root exudates of the same varieties of sesame grown in the bioreactor and in flask cultures is shown in (Figure 65).

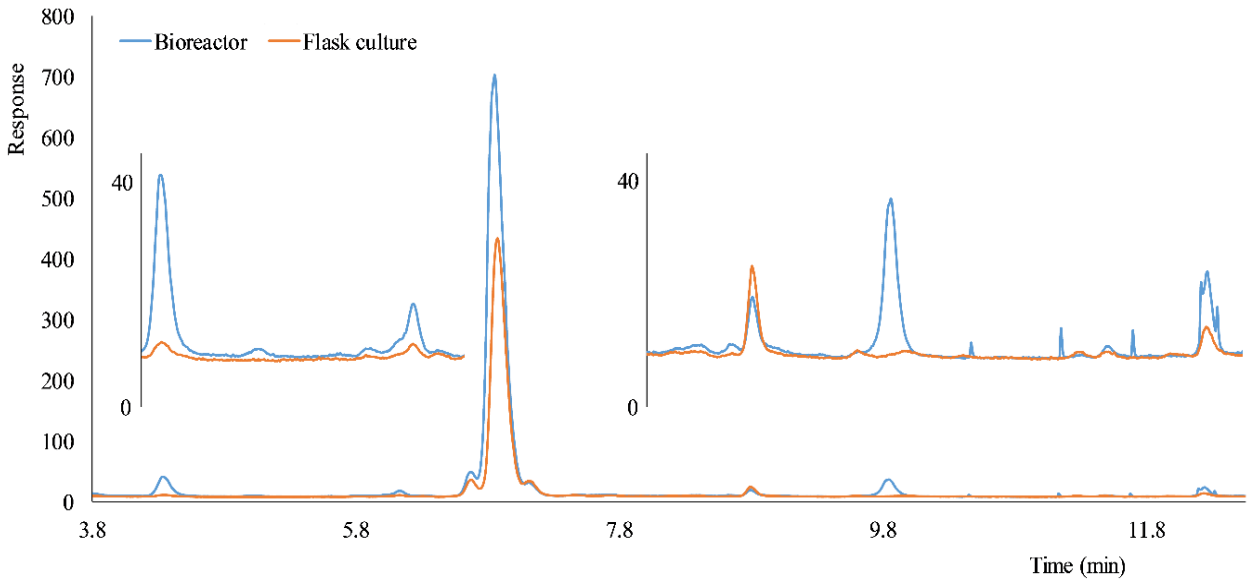

Figure 65: Comparison of HPLC-ELSD chromatograms of exudates of hairy roots of *Sesamum indicum* (Japan varieties) grown in the bioreactor and flask cultures.

HPLC-ELSD signals obtained in the analysis of hairy root exudates collected from both bioreactors and flask cultures are shown in (Table 24) and (Figure 65). All signals, except one, were detected in all three replicates of each variety, but the composition of exudates varied among varieties of the same species as well as between the bioreactor and flask cultures of hairy roots generated from the same plant varieties.

Hyoscyamus niger data were selected to facilitate the visual assessment of differences among varieties and the effect of the cultivation system on the chemical diversity of root exudates. The

data shown in (Table 24) were converted into an absence/presence and non-metrical multidimensional scaling (nMDS), which was used to ordinate the profiles obtained for each sample in two dimensions; the results are shown in (Figure 66).

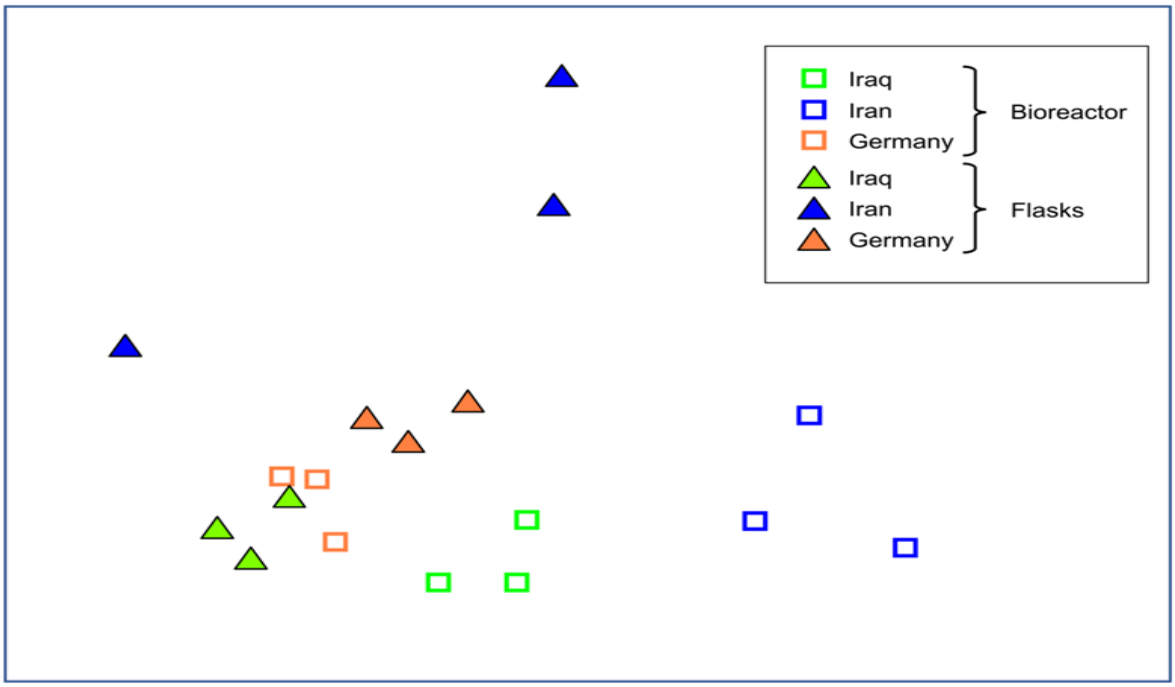

Figure 66: Non-metrical multidimensional scaling analysis of metabolic profiles of hairy root exudates of *Hyoscyamus niger* grown in bioreactors and flask cultures. Metabolic profiles analyzed by HPLC-ELSD (Supplementary Table 29) were converted into a presence/absence matrix, and the binary data was subjected to mMDS ordination. The 2D-stress was 0.09 (Diagram created by Dr. Franz Hadacek).

The nMDS analysis showed that both plant varieties and cultivation techniques were clearly separated from each other. The analysis was based on the presence/absence of metabolites, but apart from qualitative differences among the metabolic profiles, the metabolites exhibited a large difference in their abundance (Table 24). Therefore, pairwise Euclidean distances between metabolic profiles of *H. niger* varieties were calculated for each cultivation method and the distances between metabolic profiles obtained by the two cultivation methods for each *H. niger* varieties. The results were shown in (Figures 66, 67, and 68).

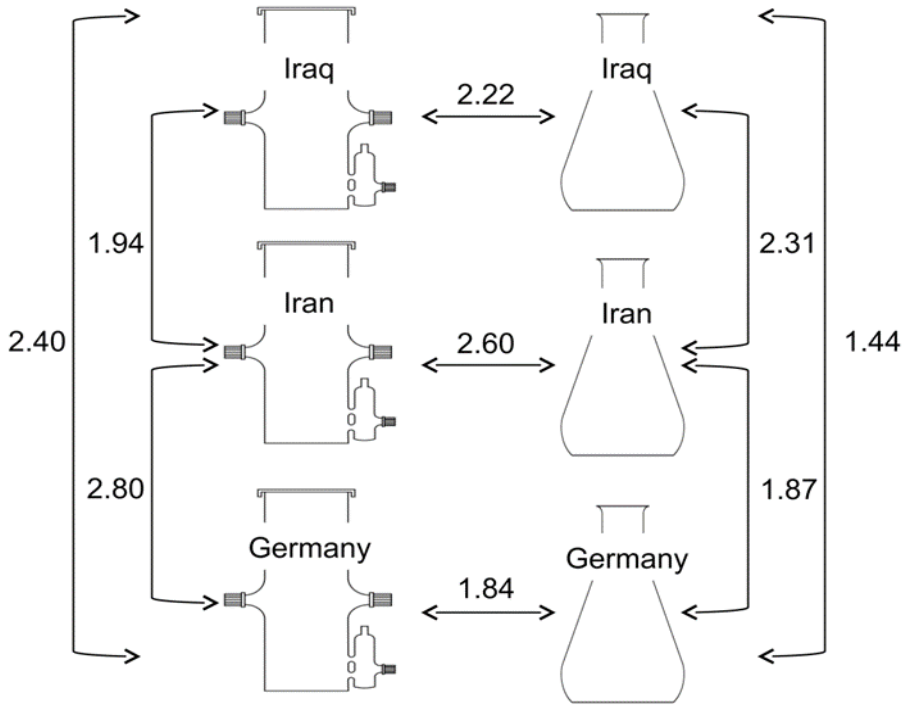

Figure 67: Euclidean distances between metabolic profiles of hairy root exudates of three varieties labeled (Iraq= KRI Daray Mar), (Iran= Iran Takhte), and (Germany= Germany 1) of *Hyoscyamus niger* grown in bioreactors and flask cultures (Diagram created by Ms. Ling Su).

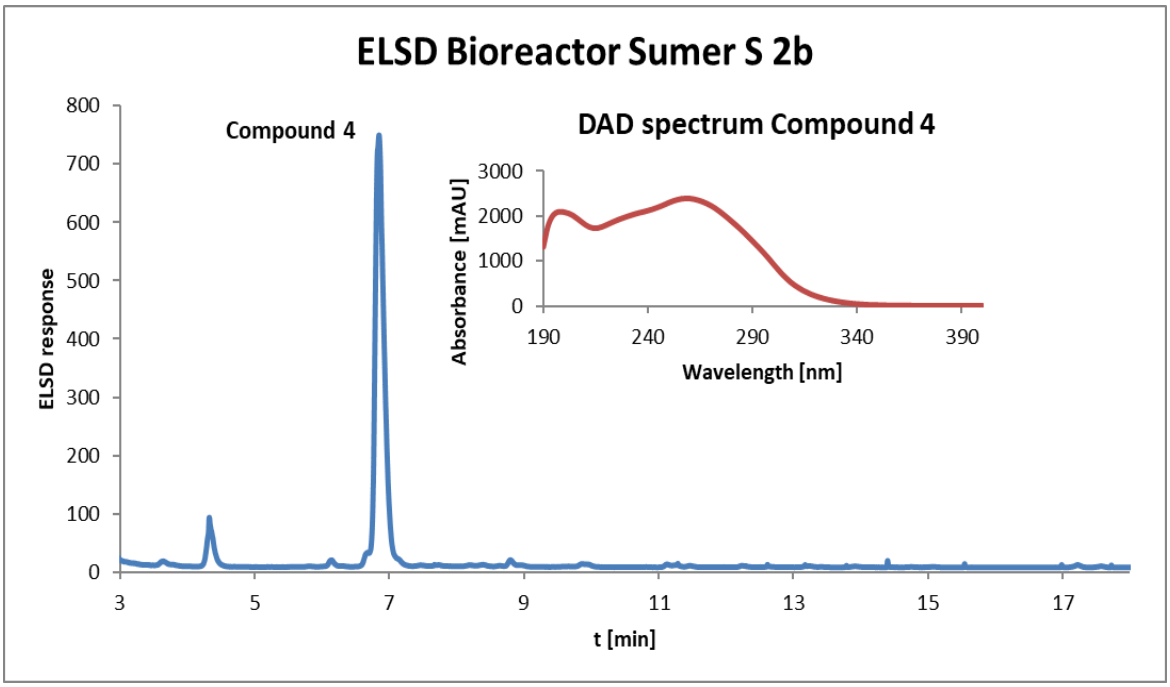

Figure 68: HPLC-ELSD peaks obtained from the analysis of hairy root exudates accumulated in bioreactor showing compound number 4

4.7.1.1. Nontargeted Analysis of Hairy Root Exudates in *Hyoscyamus niger* Bioreactor Compared to Shaking Flasks

The results of the analysis of hairy root exudates by HPLC-ELSD are shown in (Table 24) The average and standard deviation of the intensity of HPLC signals in exudates obtained from bioreactors was 20%, compared to the average of 28% for exudates from shaken flasks. Thus, the reproducibility of the composition of hairy root exudates collected in bioreactors appears higher than the reproducibility of exudates collected in shaking flasks. The total intensity of HPLC-ELSD signals detected in exudates from bioreactors (average total per varieties of 9024) was 1.6-times more than the total intensity of signals from shaking flasks (average total per varieties of 5692), indicating that hairy roots exudate more amounts of metabolites in bioreactors as compared to shaking flasks. Overall the secondary metabolite accumulation in hairy root exudate from the bioreactor is higher in the quantity and expectance. While in (KRI Daray Mar) variety the compound number 2, 10, 13 exists in the flask culture but not in Bioreactor, vice versa for the compound number 3.

Table 24: Phytochemical compounds peak area ±SD detected by HPLC-ELSD in hairy roots exudates of *Hyoscyamus niger* in bioreactor and flask cultures.

Varieties Metabolites	RT *	Iran Takhet **		Germany **		Daray Mar **	
		Bioreactor	Flask	Bioreactor	Flask	Bioreactor	Flask
Compound 1	3.6	N.d.	**224 ± 191**	N.d.	N.d.	N.d.	N.d.
Compound 2	4.3	**41 ± 4**	N.d.	**41± 4**	N.d.	N.d.	**100 ± 125**
Compound 3	6.1	**128 ± 18**	**20 ± 2**	**24 ± 1**	**32 ± 2**	**30 ± 4**	**41 ±2**
Compound 4	6.8	**12,500 ± 600**	**2300 ± 00**	**5700 ± 490**	**5100 ± 200**	**7000 ± 300**	**5900± 200**
Compound 5	7.7	**29 ± 5**	N.d.	N.d.	N.d.	N.d.	N.d.
Compound 9	9.5	**40 ± 8**	N.d.	N.d.	N.d.	**35 ± 9**	N.d.
Compound 10	9.7	**75 ±29**	**750 ±350**	**1300 ± 650**	**485± 175**	N.d.	**1750 ± 490**
Compound 11	9.9	**42 ± 29**	N.d.	N.d.	N.d.	N.d.	N.d.
Compound 12	10.5	N.d.	N.d.	**32.2 ± 2.7**	**52 ± 13**	N.d.	**120 ± 30**
Compound 13	11.1	N.d.	N.d.	**51± 18**	**110 ± 41**	N.d.	**67 ± 2**
Compound 14	11.3	N.d.	N.d.	N.d.	N.d.	N.d.	N.d.
Compound 17	14.8	N.d.	**87± 8**	N.d.	N.d.	N.d.	N.d.

* Retention time [min]. ** Signal intensity (peak area) as mean ± s.d.

4.7.2. Lignan profiling

The methanol extracts of seed, root, and hairy root from 25 varieties of *Sesamum indicum* were prepared and used for chemical analysis, and the identification of lignan in these varieties of *Sesamum indicum* using HPLC-MS phytochemical screening (Table 25) showed the presence of a different class of lignans, (Sesamin, Sesamolin, Sesaminol, Pinoresinol, and Sesamol the supplementary Appendix C.

Table 25: Mean lignan content (mg/g) in the seeds of sesame variates.

Cultivars	Sesamin	Sesamolin	Sesaminol	Pinoresinol	Total Lignans
India 1	0.75 ± 0.04	0.86 ± 0.04	0.30 ± 0.02	0.29 ± 0.05	2.2
India 3	1.70 ± 0.09	1.39 ± 0.06	0.64 ± 0.03	1.69 ± 0.13	5.42
India 7	1.48 ± 0.19	0.93 ± 0.04	0.44 ± 0.02	0.36 ± 0.04	3.21
India 8	1.68 ± 0.23	1.59 ± 0.02	0.85 ± 0.03	1.61 ± 0.20	5.73
Korea 1	1.73 ± 0.27	1.26 ± 0.07	0.24 ± 0.02	1.09 ± 0.25	4.32
Turkey	1.85 ± 0.16	1.74 ± 0.03	0.21 ± 0.03	0.11 ± 0.02	3.91
Syria	1.44 ± 0.22	0.37 ± 0.01	0.19 ± 0.04	0.08 ± 0.01	2.08
Sudan 2	1.19 ± 0.18	0.76 ± 0.01	0.12 ± 0.01	0.13 ± 0.03	2.2
Africa 3	0.94 ± 0.10	1.01 ± 0.01	0.09 ± 0.01	0.05 ± 0.01	2.09
Japan 2	1.75 ± 0.24	1.36 ± 0.02	0.15 ± 0.02	0.13 ± 0.03	3.39
China 2	1.39 ± 0.13	1.38 ± 0.02	0.34 ± 0.05	0.19 ± 0.02	3.3
Vzla 51	1.19 ± 0.14	1.33 ± 0.03	0.23 ± 0.04	0.32 ± 0.16	3.07
UCV-1	1.50 ± 0.22	1.33 ± 0.03	0.17 ± 0.03	0.44 ± 0.07	3.44
Maporal	1.28 ± 0.10	0.75 ± 0.01	0.44 ± 0.14	0.32 ± 0.04	2.79
Inamar	1.57 ± 0.12	1.08 ± 0.01	0.43 ± 0.12	0.62 ± 0.08	3.7
UCLA-1	1.61 ± 0.14	0.96 ± 0.01	0.18 ± 0.07	0.27 ± 0.14	3.02
Sumer S1a	0.77 ± 0.03	0.70 ± 0.01	0.07 ± 0.01	0.06 ± 0.03	1.6
Sumer S1b	0.49 ± 0.04	0.71 ± 0.01	0.08 ± 0.01	0.12 ± 0.03	1.4
Sumer S2a	1.47 ± 0.20	0.80 ± 0.04	0.11 ± 0.02	0.12 ± 0.01	2.5
Sumer S2b	1.98 ± 0.07	0.91 ± 0.01	0.13 ± 0.02	0.15 ± 0.01	2.66
KRI local Hawler 1	2.10 ± 0.16	1.06 ± 0.04	0.24 ± 0.04	0.17 ± 0.01	2.94
KRI local Ranya 1	2.11 ± 0.21	0.90 ± 0.02	0.20 ± 0.02	0.10 ± 0.01	2.67
KRI local Hawler 2	2.09 ± 0.14	0.94 ± 0.01	0.22 ± 0.02	0.12 ± 0.02	2.75
KRI local Ranya 2	1.92 ± 0.15	0.84 ± 0.02	0.11 ± 0.03	0.08 ± 0.02	2.5
Iran 1	1.59 ± 0.12	1.01 ± 0.03	0.08 ± 0.02	0.08 ± 0.03	2.64

All values represent means of 3 replicates ± SD.

The average lignan component in the seeds of sesame varieties indicates that the highest Sesamolin content was recorded in (Turkey) accession (1.74 mg/g) while the lowest was in (Syria) accession (0.37 mg/g) (Table 25). The result from the present study showed that from the different classes of tested lignans, the highest was Sesamin in KRI local Ranya 1 (2.11 mg/g), and the lowest content recorded was Pinoresinol in (Africa 3) accession (0.05 mg/g). The results showed that the total lignan contents of sesame varieties ranged from 1.4 to 5.42 mg/g. The highest sesamin, sesamolin, sesaminol, and pinoresinol content in sesame varieties seeds ranged from 2.11, 1.74, 0.85, and 1.69 mg/g, respectively, moreover the sesamolin content from all the 25 varieties were presented in (Figure 69).

The Sesamin content in the seed and hairy root was higher than the normal root, the highest Sesamin content in the root was recorded in KRI local Ranya 2 accession (225 mg/kg), and from the hairy root was recorded in Inamar varieties (75 mg/kg) while from the seed was recorded in Iran 1 accession (17 mg/kg) (Figure 70).

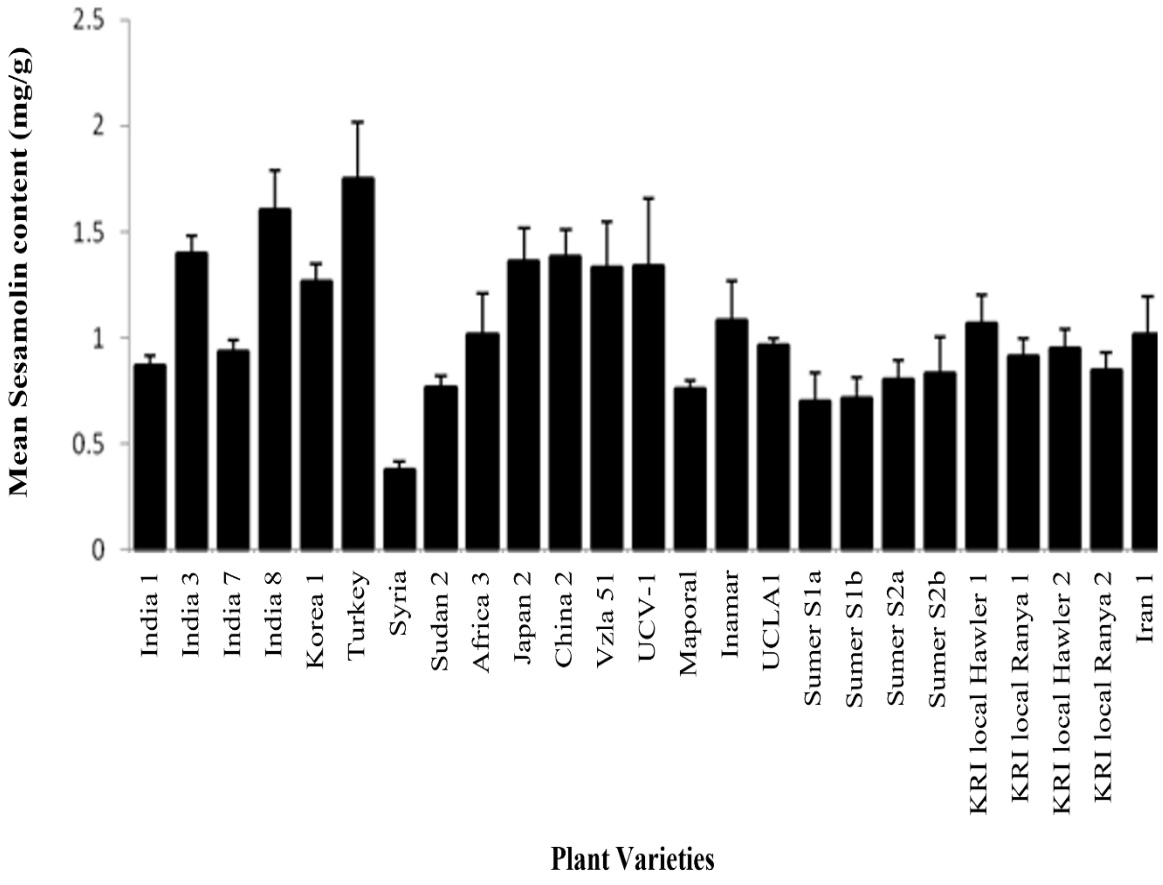

Figure 69: Distribution of seed Sesamolin content (mg/g) in the twenty-five sesame varieties.

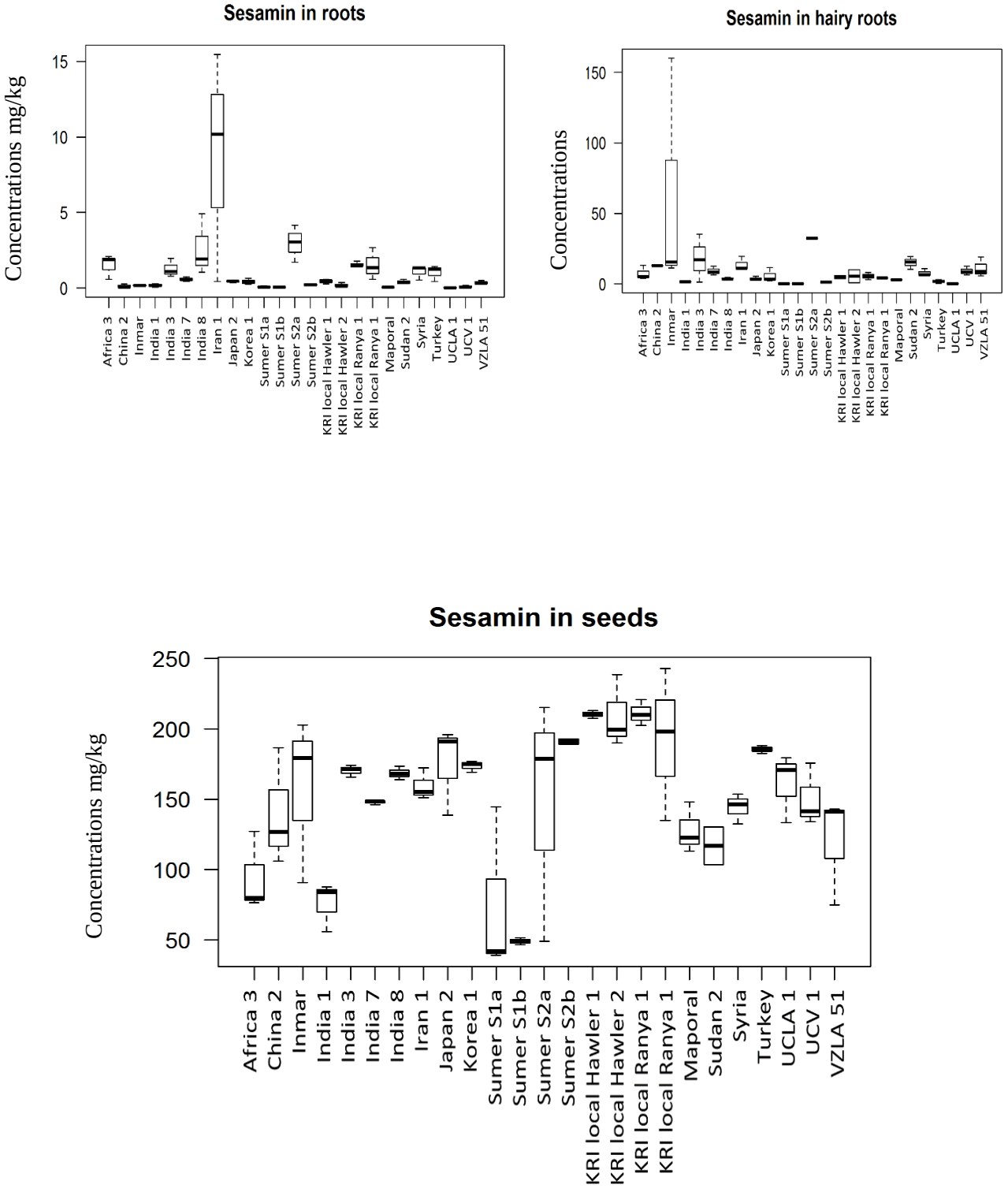

Figure 70 : Statisticalplots show the lignan (Sesamin) content of 25 S. indicum varieties within Root, Hairy root, and seeds.

4.8. DNA Sequencing

4.9. Amplification of the DNA Fragments

The extraction method, which followed in this research showed a good quantity and pure DNA when checked on gel electrophoresis (Figure 71a). A direct method was performed in this study for amplifying the fragments of various genes in *Sesamum indicum* and *Hyoscyamus niger*. By using six specific primers, six genes were amplified from the *Sesamum indicum.* The genes were *FAD2, SAD, FAD1, GBssr-sa-108, GBssr-sa-182* and *GBssr-sa-184* which had a size 647, 428, 782, 183, 232 and 175 bp, respectively. The amplified products are shown in (Figure 71b and c). According to the results, only *FAD2* and *SAD* primers amplified an obvious product in all the samples of *Sesamum indicum*. Therefore, only those samples amplified by these two primers were sequenced.

4.10. Multiple Sequence Alignment

The sequence data were obtained from amplified *HNTR1,* and *HNTR2* genes in *Hyoscyamus niger* were compared with the reference sequence (GenBank varieties no.) AB026544 and AB02654, respectively (Figure 72 and 73). Moreover, both amplified *FAD* and *SAD* genes in *Sesamum indicum* were compared with the reference sequence AY770501.1 and XM_011078814.2, respectively (Figures 74 and 75). All the sequence data were successful without trimming. The length of the sequence was varied according to the quality of the amplified PCR products, and the sequence data were obtained.

The alignment is done by online http://multalin.toulouse.inra.fr software. The alignment shows the variations in different colors. Some variations were detected only among different samples from the same region. Moreover, a significant difference was observed among all the sequences collected from different locations.

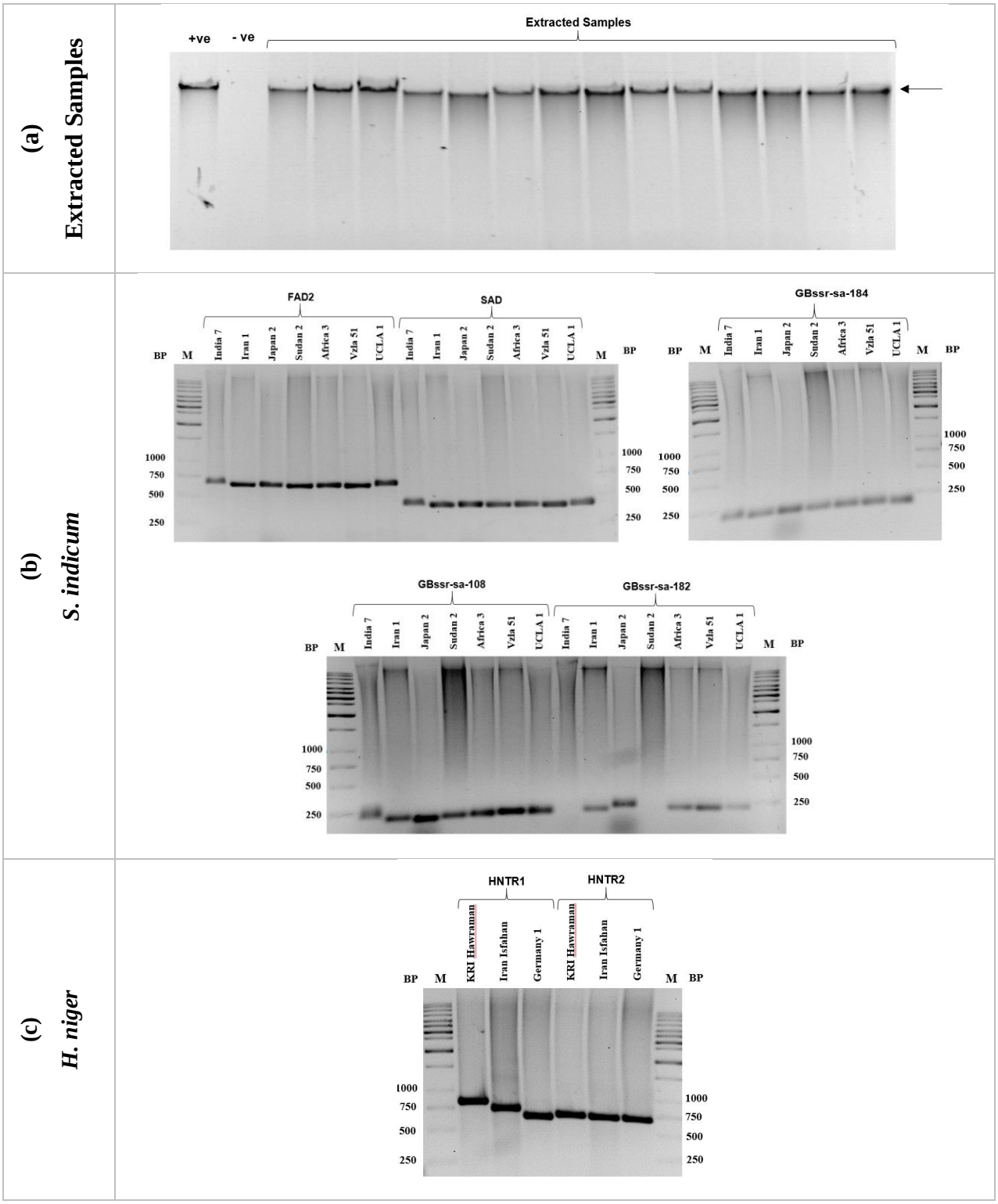

Figure 71: Gel electrophoresis showed extracted genome and amplified PCR products. a) the gel showed some extracted samples. b) selected genes amplified from *S. indicum*. c) selected genes amplified from *H. niger*. The marker (1Kb) showed in the Base pair.

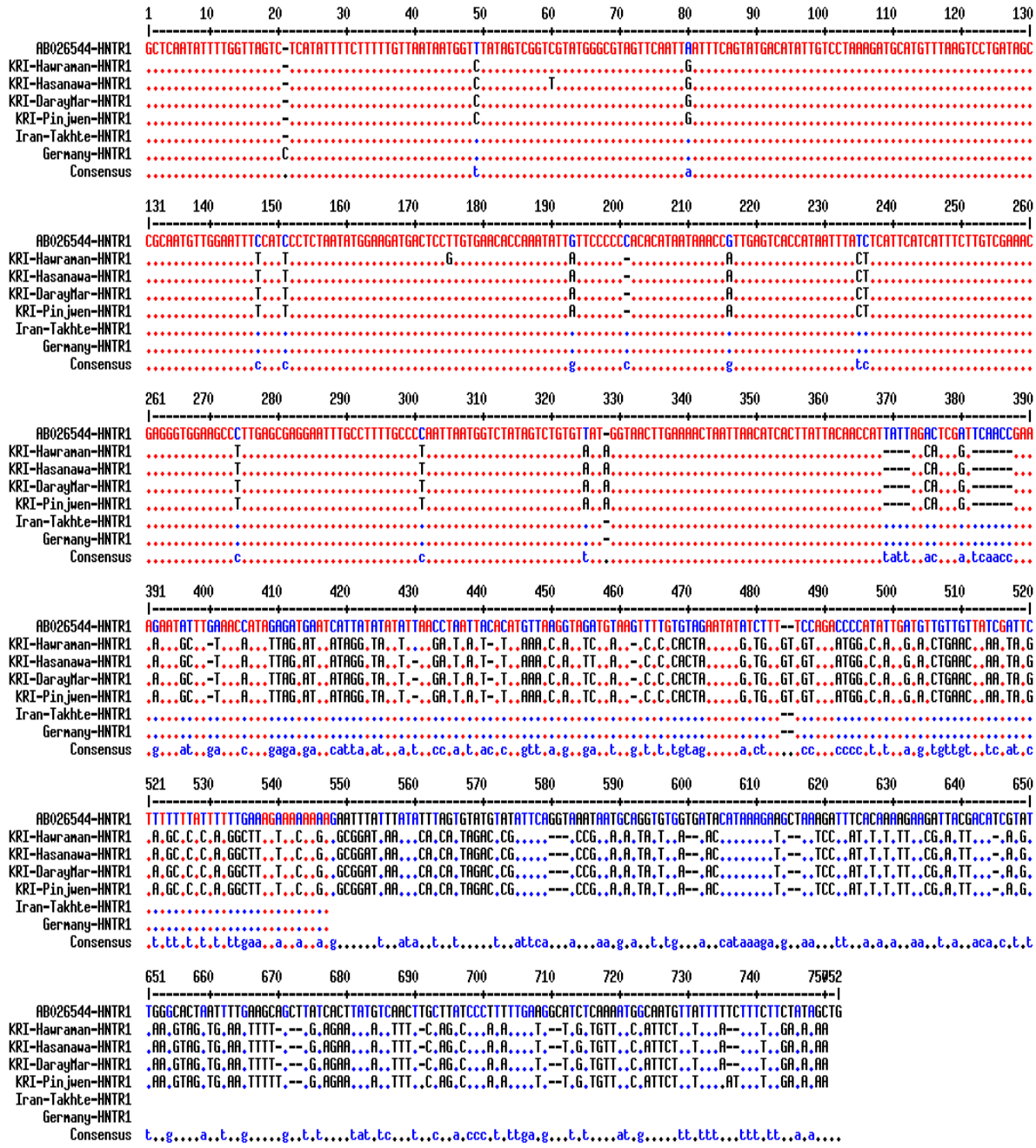

Figure 72: Multiple Sequence Alignment of the *TR1* gene in *Hyoscyamus niger* based on Nucleotide Residue. The figures represent the alignment of the six samples collected from Germany, Iran, and Iraq. The alignment was performed in an online http://multalin.toulouse.inra.fr software. The numbers on the top are showing the positions of each nucleotide and done by the software. The words *TR1* in the name of each sample refers to the Tropinone reductase one gene in *Hyoscyamus niger*, while the numbers refer to the personal enumeration for each sample. The similarity showed by a dot (.), the variation shows by the different colors. The red, blue, and black colors represent High consensus, Low consensus, and Neutral nucleotides, respectively.

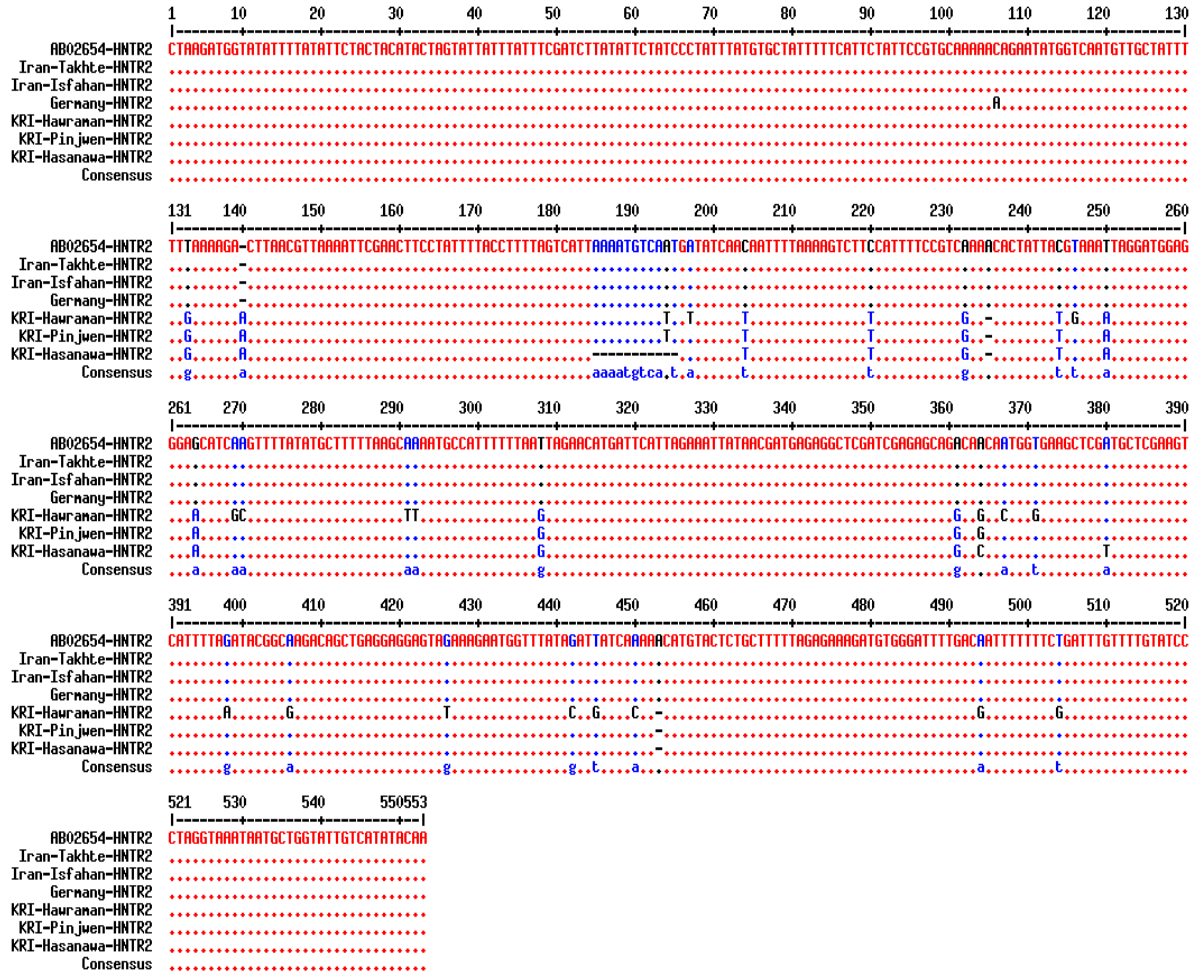

Figure 73: Multiple Sequence Alignment of the *TR2* gene in *Hyoscyamus niger* based on Nucleotide Residue. The figures represent the alignment of the six samples collected from Germany, Iran, and Iraq. The alignment was performed in an online http://multalin.toulouse.inra.fr software. The numbers on the top are showing the positions of each nucleotide and done by the software. The words *TR2* in the name of each sample refers to the Tropinone reductase two genes in *Hyoscyamus niger*, while the numbers refer to the personal enumeration for each sample. The similarity showed by a dot (.), the variation shows by the different colors. The red, blue, and black colors represent High consensus, Low consensus, and Neutral nucleotides, respectively.

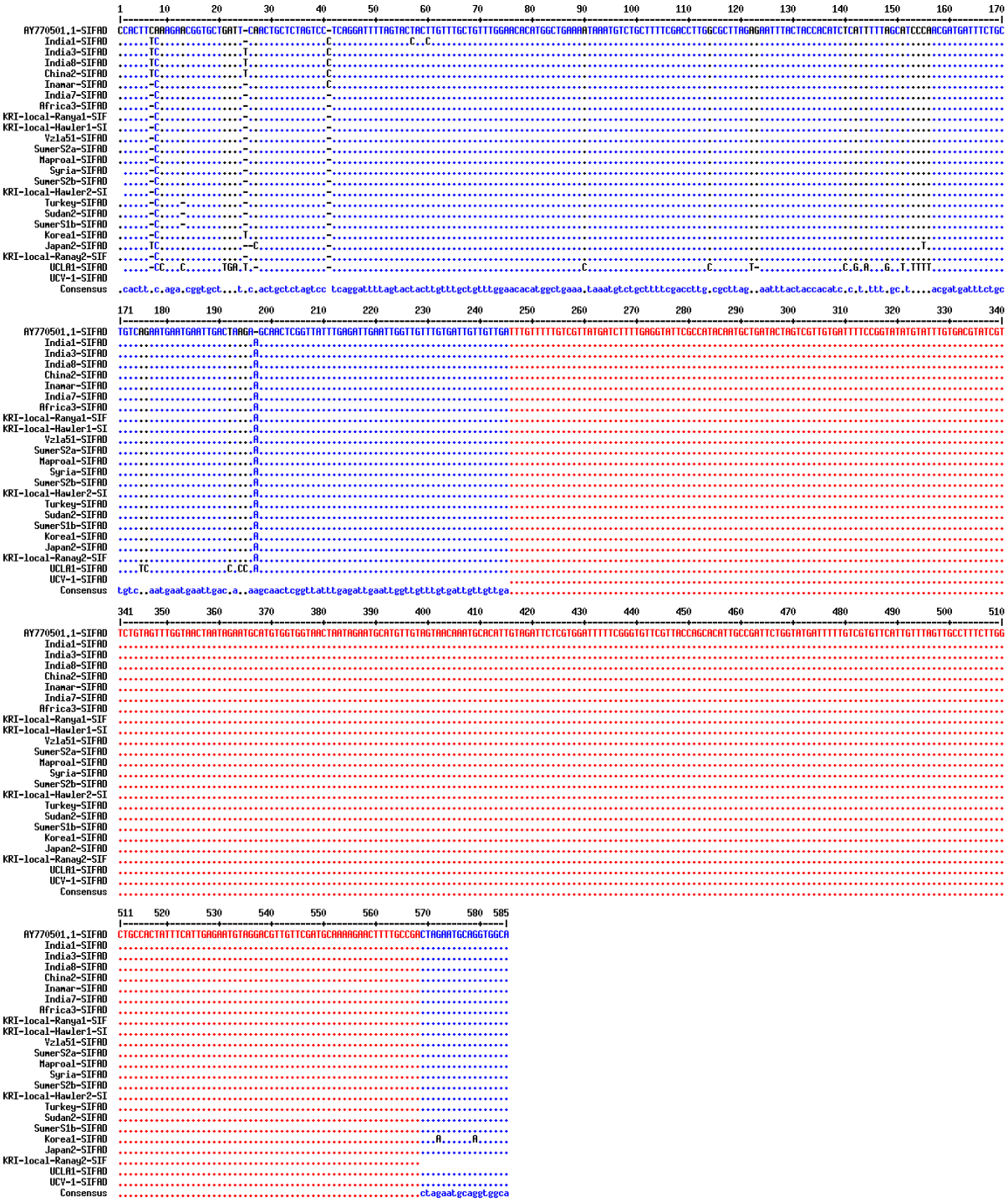

Figure 74: Multiple Sequence Alignment of the *FAD* gene in *Sesamum indicum* based on Nucleotide Residue. The figures represent the alignment of the 24 samples collected from different locations in comparison with the reference AY770501.1. The alignment was performed in an online http://multalin.toulouse.inra.fr software. The numbers on the top showing the positions of each nucleotide. The words *FAD* in the name of each sample refers to the fatty acid desaturase gene in *Sesamum indicum*, while the numbers refer to the personal enumeration for each sample. The similarity showed by a dot (.), the variation shows by the different colors. The red, blue, and black colors represent High consensus, Low consensus, and Neutral nucleotides, respectively.

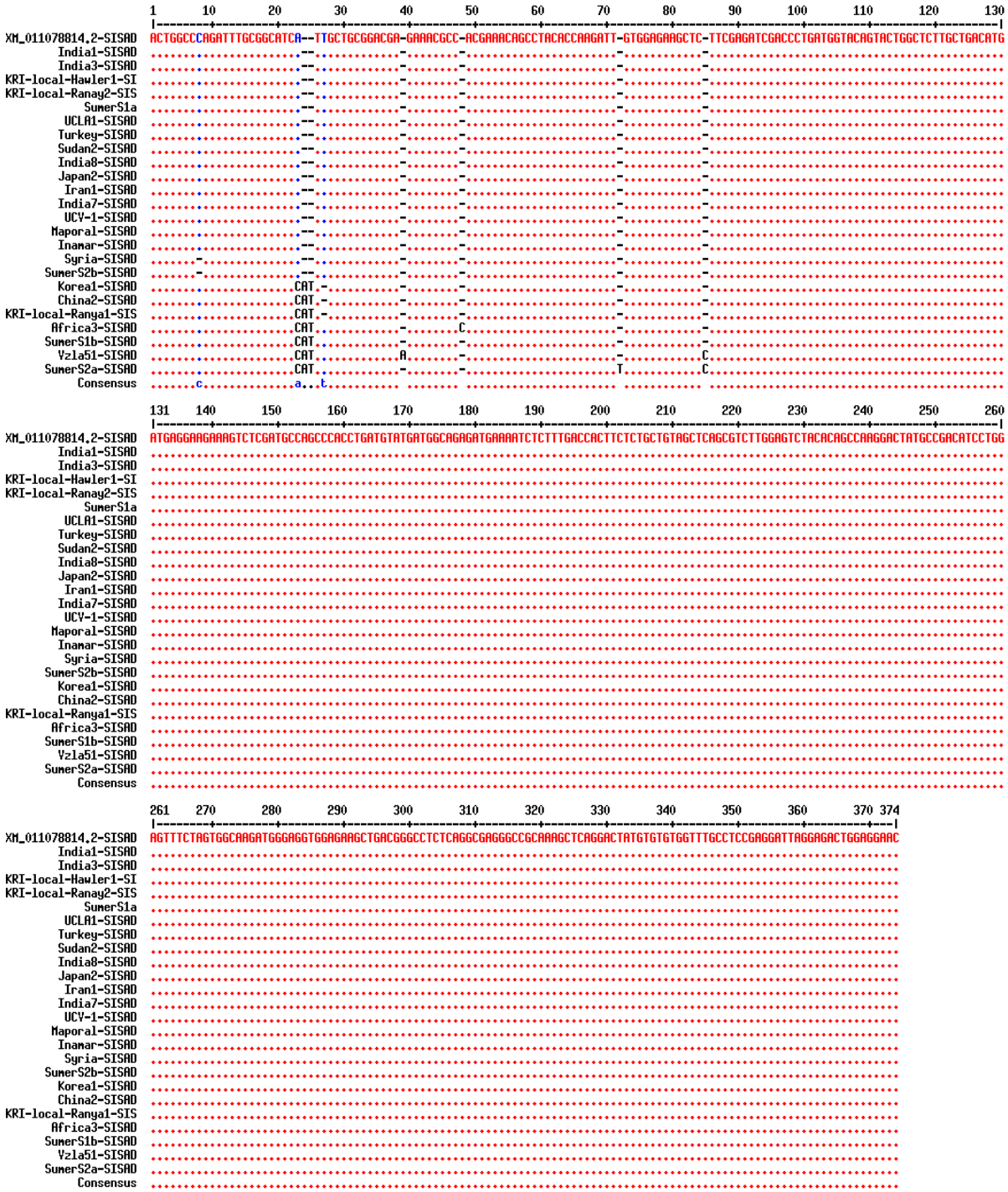

Figure 75: Multiple Sequence Alignment of the *SAD* gene in *Sesamum indicum* based on Nucleotide Residue. The figures represent the alignment of the 24 samples collected from different locations in comparison with the reference XM_011078814.2. The alignment was performed in an online http://multalin.toulouse.inra.fr software. The numbers on the top showing the positions of each nucleotide. The words *SAD* in the name of each sample refers to the stearoyl-acyl desaturase gene in *Sesamum indicum*, while the numbers refer to the personal enumeration for each sample. The similarity showed by a dot (.), the variation shows by the different colors. The red, blue, and black colors represent High consensus, Low consensus, and Neutral nucleotides, respectively.

4.11. Phylogenetic Construction

The genetic relationships between *H. niger* varieties were examined using phylogenetic analysis, which was carried out by MEGA7.0 (Figure 76 to 79). The tree was assessed by the neighbor-joining method to estimate the genetic evolution of the generated datasets. The distance in the tree was computed under a Kimura 2-parameter model and was in the units of the base number substitutions per site. Statistical analysis was done using the bootstrap approach via 100 replications that were generated pseudo-replicates nucleotide alignments from the sites in the original alignment. For each replicate, the tree generated, and the method was repeated 100 times and a percentage score giving for the same grouping.

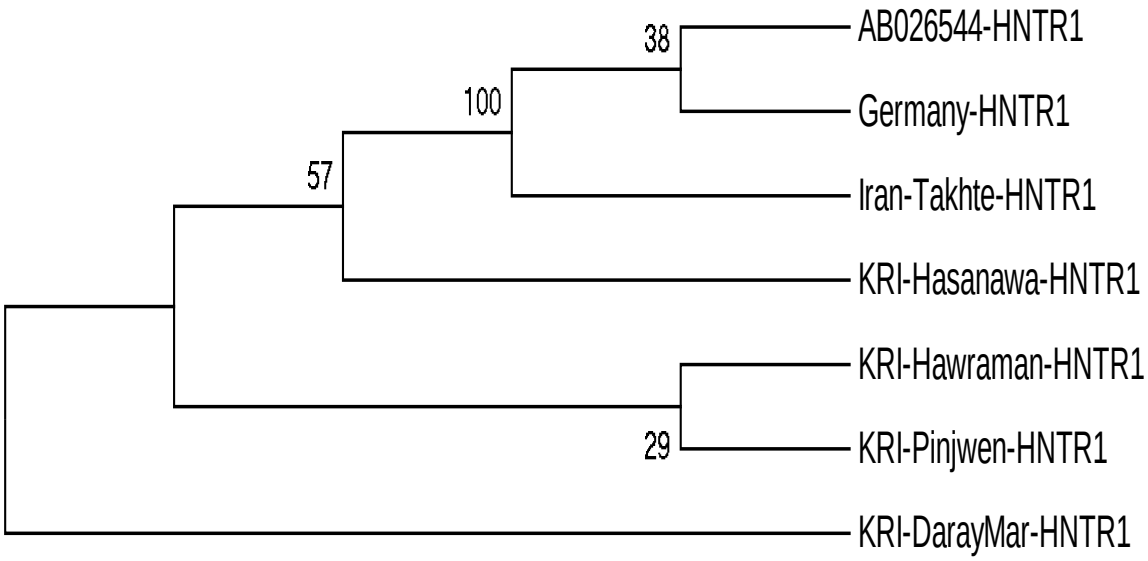

Figure 76: Evolutionary relationships of *TR* 1 gene amplified from *Hyoscyamus niger*. The evolutionary history inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to dividers reproduced in less than fifty percent bootstrap replicates are collapsed. The percentage of duplicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were calculated using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per position. The analysis involved seven nucleotide sequences. Codon positions included was 1st+2nd+3rd+Noncoding. All positions containing gaps and absent data were eliminated. There was a total of 525 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

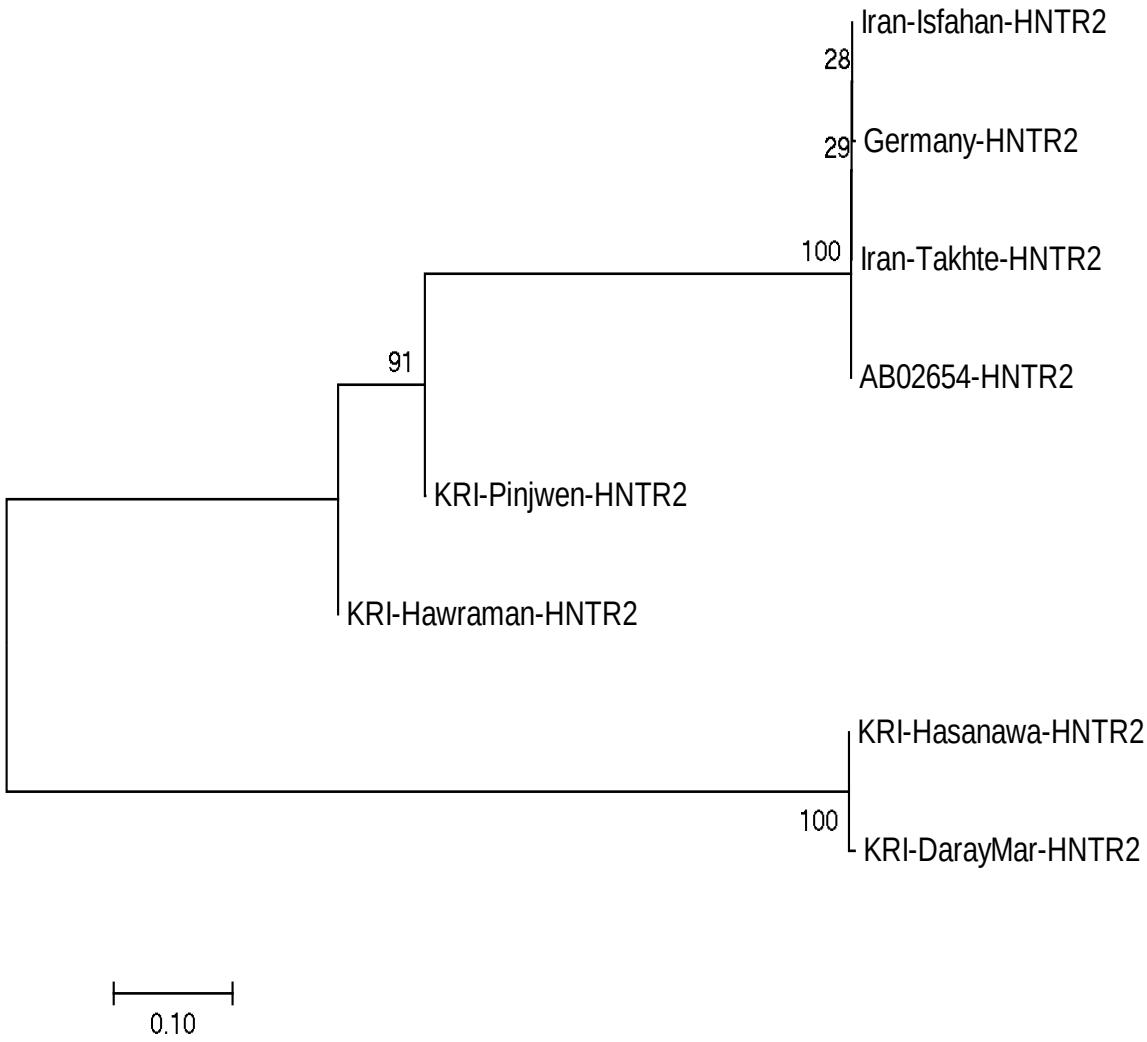

Figure 77: Evolutionary relationships of *TR* 2 gene amplified from *Hyoscyamus niger*. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.42789783 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved eight nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 540 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

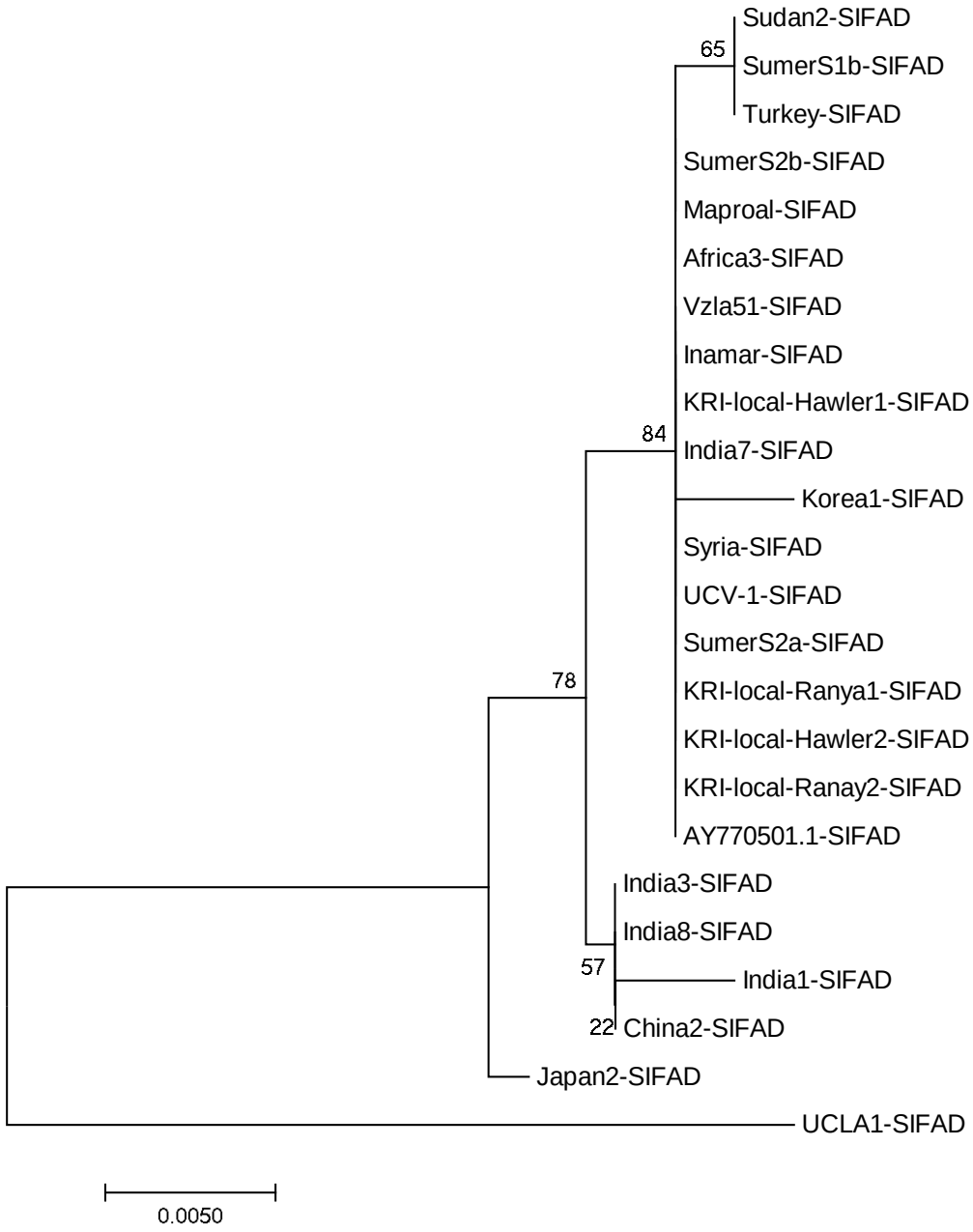

Figure 78: Evolutionary relationships of the *FAD* gene in *Sesamum indicum*. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.05357005 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 24 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 577 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

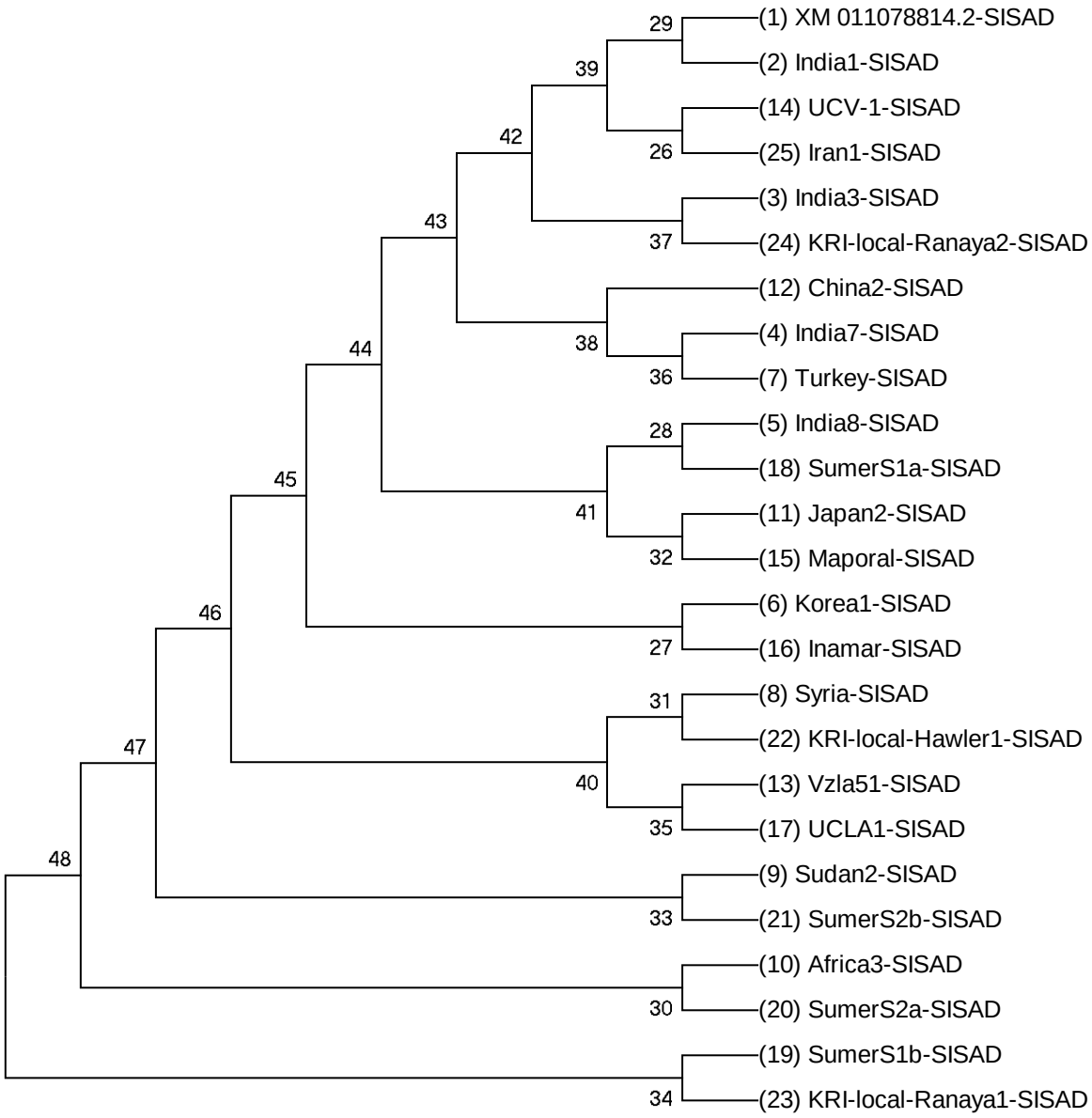

Figure 79: Evolutionary relationships of the *SAD* gene in *Sesamum indicum*. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 367 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

CHAPTER FIVE

5. DISCUSSION

5.1. Seed sterilization

In the current study, *Hyoscyamus niger* and *Sesamum indicum* seed surface sterilization were successfully achieved using 70% ethanol for 30 seconds followed by 6% commercial Clorox (5% sodium hypochlorite) for 15 minutes they were good sterilant and effective in overcoming contamination (Gopal *et al.*, 1998). Many reports regarding sterilization of explants under *in vitro* conditions using Clorox have been reported, depending on exposure duration in which longest duration may lead to the death of cells and decrease the viability of the plant cells (Kumar *et al.*, 2015).

These study results correspond with *H. niger* and *S. indicum* seed and leaves, which also showed an effective role of 70% ethanol for 30 seconds and 6% commercial Clorox for 15minutes in reducing contamination rate and keeping the viability of the seeds. Sodium hypochlorite contains chlorite, which is extremely toxic due to high bleaching action, which combines strongly with protein causing the death of the organism (Ron *et al.*, 2014).

5.2. Seed germination

In the current study, GA3 was used to stimulate the germination in all seeds when compared to the control treatment. Gibberellin stimulates aleurone proteins to synthesize and secrete α-amylase and other hydrolase starch leading to cell elongation and seedling development (Woodger *et al.*, 2010). Moreover, an effective indicator of the degree of dormancy in plant seeds is the α-amylase enzyme activity (Vieira *et al.*, 2002). Germination depends on the elimination of the seed coat that has been facilitating through chemical scarification, providing support for GA3's simulator role in germination.

Enhancing effects of GA3 are observed in the present study with the role of darkness in the primary steps. The GA3 treated seeds gave the highest germination rates compared with untreated controls under the same conditions. Results showed that the lowest germination percentage was recorded in control untreated seeds (Figure 4.25). The seeds from KRI Darae-Mar and Germany 1 showed the best response to GA3, while the seeds from *S. indicum* varieties mostly showed a similar response to GA3. The germination response between the control untreated and GA3 treated *S. indicum* seeds was somehow similar because the *S. indicum* seed dormancy is relatively low comparing to *H. niger* seeds. The impact of GA3 as a promoter was

supposed to boost when treated with chilling. GA3 could increase the embryo's development capacity and supplies food from endosperm as a result of hydrolytic enzymes (da Silva, Toorop, Nijsse, Bewley, & Hilhorst, 2005). The results showed that *H. niger* and *S. indicum* seeds had a partial dark requirement to germinate.

Gibberellins are hormone classes most obviously involved in seed germination control and advancement. The levels of these compounds in seeds were comparatively large but were generally smaller in mature dormant seeds, especially dicotyledonous. Endogenous gibberellins can overcome different types of dormancy, including physiological dormancy, photo dormancy, and thermo-dormancy (Seiler, 1998). In this study, GA3 considerably improved the germination rate based on the dose that was used to suggest physiological dormancy associated with partly dormant embryos. The fact that 250 mg / L GA3 provided the greatest germination rate proved during this study.

5.3. Hairy Root Culture

5.3.1. Effect of Different Sterilisation Methods on Explant Viability

The sterilization method is the removal of contaminants that exist on the surface of explants without damaging the tissue of the plants. Various surface sterilization techniques and many separate surface sterilizers were used as well. The usual chemicals used for surface sterilization was sodium hypochlorite, ethanol, calcium hypochlorite, and mercuric chloride. Contamination caused by the environmental surroundings organisms and improper sterilization. Contaminations can lead to making plant development slow, tissue necrosis, decreased shooting, and less rooting and decrease the viability of the plant tissue (Odutayo *et al.*, 2007). Protocol and time given for explants surface sterilization were very important factors that depend on the maturity and the used tissue type.

In the present study, an aseptic protocol for growing the seeds from both plant species and varieties by using sodium hypochlorite and ethanol for seed sterilization and growing under aseptic conditions in MS medium until the initiations of cotyledon and plant leaves, then using these explants for hairy root induction. This protocol ensures the viability of the explants and decreases the necrosis with a very low rate of contamination; this is because the seed coat gives the highest protection to the seed comparing to other plant parts. While non-aseptic protocol, in which the explant (any part of the plant such as leaves, stems fruit) was directly exposed to the sterilizer, then has been used for hairy root induction. This long protocol exposure mainly led to necrosis, and the viability decrease, while short exposure mainly led to high contamination.

5.3.2. The efficiency of Different *A. rhizogenes* Strains on Hairy Root Induction

Agrobacterium rhizogenes strains promoted a positive response, and the development of hairy root also extremely demonstrated strong growth with the accumulation of a wide variety of secondary metabolites. The results showed that the response of *H. niger* and *S. indicum* is differed according to different strains of *A. rhizogenes.* the results showed ATCC15835 was more successful than A4 in its transformation potential and hairy root induction.

Different *A. rhizogenes* strains had a different effect on the morphology, growth rate of hairy roots clones, and secondary metabolite production. Hairy roots are important in selecting a clone for further study to achieve commercial production phytochemicals in vitro. In this manner, selecting the most effective strain of *A. rhizogenes* was one of the most critical steps to obtain successful hairy root induction and proliferation.

The significant difference of the hairy root clone's growth rate, induced by ATCC15835 and A4 *A. rhizogenes* strain, could be due to the difference in the copy number of interested DNAs into the genome of different clones or differences in T-DNA gene expression patterns in various hairy roots (Archana Giri and Narasu, 2000). The genetic stability of hairy roots *H. niger* and *S. indicum* has advantages of achieving commercial production of alkaloids and lignans *in vitro*, then biomass production for a long period without a significant decrease in yield (Zolala *et al.*, 2007).

Two major factors that influence secondary metabolites *in vitro* production include the biomass generated over a given period and the number of metabolites synthesized per unit of biomass. The results showed that the growth rate of the hairy roots generated by using *A. rhizogenes* ATCC15835 was higher than that of A4 strains (Figure 32). The result obtained during his study showed significant differences in hairy root inductions and biomass accumulation between the varieties of the same plant varieties using ATCC15835 and A4 species. The transformation of *H. niger* and *S. indicum* was 100% toward *A. rhizogenes* ATCC15835 also; there was some similarity between the varieties of the same plant species for hairy induction. *A. rhizogenes* A4, results showed variability even between the varieties of the same plant species with almost half potential compared to ATCC15835.

Hairy root initiation and gene transfer in *H. niger* and *S. indicum* were affected by the used *A. rhizogenes* strains. Particularly, the genes involved in the metabolic pathway of secondary metabolite biosynthesis. The previous transcript analysis study clearly showed that individual gene expression strain-specific. Overall, selecting and identifying an appropriate strain of *A.*

rhizogenes was helpful for enhancing secondary metabolite production and hairy root biomass production.

5.3.3. Hairy Root Growing in Liquid Medium (Shaking Flask culture)

Hairy roots from *H. niger* and *S. indicum* varieties induced by the two strains of *A. rhizogenes* ATCC15834 and A4 were sub-cultured to fresh solid MS medium and after necessary growth hairy roots were transferred to shake flask liquid MS medium, in complete dark condition. The rapid growth of hairy roots in the antibiotic and hormone-free liquid MS medium was observed especially, the hairy roots growth obtained after transformation with ATCC15834 strain.

Moreover, hairy roots from *A. rhizogenes* A4 strain were lower in induction and biomass accumulation while the explant produced hairy roots by ATCC15834 strain were higher in growth rate and induction. These roots were thick and had produced many lateral branches. Furthermore, newly formed hairy roots were white and later became brown.

The medium type was reported to affect HRCs growth and proliferation, and the appropriate liquid culture conditions could have a role in the biomass increasing of the hairy roots. Also, the liquid MS medium hairy root culture was faster in development and growth than the solid medium hairy roots (Huang *et al.*, 2014). The results of the biomass yield of the Hairy roots from *H. niger* and *S. indicum* showed significant differences in yield and morphology of the HRCs between even the successions of both plant species. These differences may be related to the genetic constitution of these varieties and the environmental conditions or various locations of the variety collection.

In *H. niger* and *S. indicum* hairy roots, nodular-like structures may be caused when nutrients become restricted, or the medium is not well aerated. In the meantime, the hairy root of the bioreactor did not contain this structure. The benefit of this hairy root system was that the roots were established in the medium as independent organs and can be cloned by a root tip subculture (Boisson-Dernier *et al.*, 2001). A difference in the morphology of the hairy roots was first observed in the cultivation of the roots in the liquid medium. The differences in these morphologies may depend on the type of the selected tissue for the initiation of cultures or the time of the growing cycle during which the roots have been harvested. Shaker incubator speed and liquid volume were previously demonstrated as crucial factors for oxygen transmission in shake flasks (Van Suijdam *et al.*, 1978), also The type of flask seals, whether silicone foam, aluminum foil or cotton, had only a tiny impact on the total stress of oxygen in the flask headspace (Yu and Doran, 1994).

Previous results suggested that limiting constituent affecting the growth of the hairy roots was oxygen (Kanokwaree and Doran, 1997). Our results confirmed this hypothesis in which the HRCs grew in shaking flask compared with those grown in bubble bioreactor when the oxygen supplying to the hairy roots was higher. Hairy root growth and final biomass were improved significantly using these treatments compared with the shaking flask culture.

5.3.4. Bubble Bioreactor

A simple bubble bioreactor was developed during this study based on aeration and agitation by a generated bubble, which reduced two processes in one step. Glass and plastic pads allow the normal distribution of the hairy root within the system.

This system was particularly appropriate for plant hairy root culture and secondary metabolite production and to generate active compounds with higher quality and quantity compared to the field plants. Moreover, plant HRCs were also physiologically and genetically stable.

The results from the present study showed that the dry weight biomass obtained in this bioreactor from *H. niger* and *S. indicum* plant varieties were significantly different from the shaking flask culture. Morphologically, the hairy roots were more proliferated and higher condense with bright color and juvenile (Figure 40). Furthermore, the HP-LC analysis shows higher chemical content in Bioreactor; also, there are some active chemicals found in bioreactor exudate for both plants, which did not appear in shaking flask culture. These results clearly indicate the potential of bioreactors to produce plant-derived active compounds such as pharmaceuticals and valuable chemicals. Meanwhile, the Iraq variety showed some different results in which the flask culture exudates exhibit more secondary metabolite comparing with the bioreactor; this may be related to the genetic content or integrated T-DNA.

5.4. Biomedical Activity

During the current study, the biomedical activity of *H. niger* and *S. indicum* was studied via the most advanced and accurate method by using the *Caenorhabditis elegans* model. For this purpose, a different type of plant extract is used. The methanol extract of roots, hairy roots, and seeds, essential oil, and fexed oil were used. The thermal release which is a new protocol in a biomedical activity designed and examined during this study. The biomedical activity of *H.*

niger extracts against *C. elegans* examined in this study were both qualitatively and quantitatively measured by using lethal concentrations LC_{50} and LC_{90} with Lethal temperatures LT_{50} and LT_{90}.

5.4.1. Essential oils

Different concentrations of the essential oil from three varieties from Henbane were tested by the serial broth dilution method to determine the lethal concentrations and mortality percentage. The nematocidal analysis explained that the essential oils which extracted purely using the Clevenger apparatus showed considerable activity against the *C. elegans*. This study can be considered as the first study conducted on the anti-nematode activity of *Hyoscyamus niger* essential oil; the results showed highly significant nematocidal activity compared with the manufacturer's regular antinematode medications. The cytotoxicity of essential oils depends on its components. Furthermore, an association with cell metabolism and apoptosis induction has been shown for essential oil components (Reichling *et al.*, 2009).

Essential oils include various volatile molecules, such as terpenoids and terpenes, aromatic and aliphatic phenol-derived compounds may have bactericidal, viricidal, and fungicidal effects. Essential oils directly affected the pathogens' cell membrane by increasing permeability and leakage of vital intracellular elements and ultimately disrupted the respiration of cells and the microbial enzyme system. Furthermore, due to type and concentration, they also showed the cytotoxic effects on living cells (Akthar *et al.*, 2014).

5.4.2. Methanolic Extracts of Hairy Root, Root, and Seed

Hairy roots exhibited typical transformed roots characteristics: rapid growth, lack of geotropism, and extensive lateral branching. The current study can be considered as the first study conducted on the nematocidal activity of hairy root extract of *H. niger* and *S. indicum*. The results of the antinematode analysis showed that the hairy root extract of *H. niger* exhibits significant mortality to *C. elegans,* which reached 100%, as showed in (Table 10). Meanwhile, *S. indicum* showed moderate nematocidal activity comparing with *H. niger*. The hairy root exhibited higher lethality comparing with normal plant roots. The hairy roots showed distinguished biomedical activity, which suggests either the synthesis of new constituents in hairy roots or interaction between compounds from hairy roots, which might be responsible for this activity (Wang *et al.*, 2012).

The efficiency of the hairy root methanolic extract showed to be higher than the normal root, and somehow similar to seed extract. Thus, transformed hairy roots may accumulate more

active chemicals, comparing to the normal root, a useful biotechnological tool. And considered as a good tool for obtaining higher yields of antibacterial compounds found in medicinal plants (Jain *et al.*, 2008).

The methanolic extracts of normal untransformed, lyophilized plant roots were prepared from the mature plant (Figure 23). Results indicated that the nematocidal activity of the plant root was lower compared to the hairy root and seed methanol extract. The highest mortality obtained was 75.7% for *H. niger* extracts while for *S. indicum* was 24% at 500 µg/mL This may have resulted from the accumulation of the active plant secondary metabolite in the seed and hairy roots but not the same for the normal plant root. The chemical analysis of the root and normal roots also support this idea, in which the secondary metabolite accumulation in the hairy root was significantly higher compared with normal plant root.

Both crude and flask oil extract from the seed of *H. niger* and *S. indicum* showed significant mortality against *C. elegans*. The methanol crude extracts of *H. niger* showed strong nematocidal activities against the *C. elegans,* with the mortality percentage reached 97%, and the lowest lethal concentration, which kills 50% was recorded. The extracts showed higher nematocidal activity on KRI Pinjwen varieties than the other varieties. This may due to the role of the environmental conditions of the different climate, which has a great role in secondary metabolite and active compound accumulation in the plants (Kebaili *et al.*, 2019).

The previous studies on the phytochemical analysis of *Hyoscyamus niger* confirmed that plant seed contains alkaloids, tyramine derivative, withanolides, lignan amides, and flavonoids (Dulger and Dulger, 2015). Also, several important lignans were recorded in *H. niger*, such as Sesamol, Pinoresinol, Sesaminol, Sesamin, and Sesamolin, the lignans show effective pharmacological and activity (Huiqin *et al.*, 2019). This strong biomedical activity may be relative to the presence of toxic metabolites. *Sesamum indicum* is known as an oil crop. Lately, the researchers showed the biomedical applications of this plant. The seed extract also showed a moderate nematocidal activity of 43.67%. Previous research indicated that the *S. indicum* seed methanol extract shows antimicrobial and antioxidants activities that can be caused by phenolic substances, such as lignans and other secondary metabolites. (Nigam *et al.*, 2014).

Fixed oils are known to be rich in triacylglycerol esters and also are composed of saturated and unsaturated fatty acids exhibit antimicrobial activity or maybe modulators of antibiotic activity (Tavares *et al.*, 2014). The fixed oil from *H. niger* showed significant mortality for *C. elegans* in which the highest mortality recorded was 96.00%, and for *S. indicum,* the highest mortality recorded was 53 %. The difference in biomedical activity between *H. niger* and *S. indicum*, related to the existent of different toxic alkaloids in *H. niger,* which are not found in *S. indicum* (Dulger *et al.*, 2010).

One mechanism which explains the synergistic effect of the fixed oil is, in part, that made of saturated and unsaturated lipids in its hydrophobic nature. These may interact with a dual lipid layer of the cellular membrane that damage the physiology of the microorganism, changes its respiratory chain and energy production or turn the cell mainly into exaggeration (Burt, 2004).

5.4.3. Thermal Release Anti nematode activity

Smoke from henbane was a traditional therapy for toothache and other diseases, and there are several geological proofs in Asia and around the world (Fenwick and Omura, 2015). Along with history, people tried to get a cure from the medicinal plant through different procedures. And there are many books and reviews describing these protocols, but the main problem is most of them have not been proved scientifically and cannot be practiced in modern medicine as a source for disease treatment. During this study, for the first time, we designed and proved scientifically one of the oldest methods of treatment with an important plant black henbane. Previously, in many different parts of the world, the heated release compound from *H. niger* was used to eliminate the parasitic nematode of human and the water extract of this plant to kill the parasitic nematode of the human gastrointestinal tract (Begum, 2010).

Furthermore, we designed an instrument called Thermal releaser (Figure 21) with a protocol named Thermal release. Results showed significant achievements from *H. niger* seeds. Total *C. elegans* mortality was provided at 100% for all varieties at 100°C and 94% for KRI Pinjwen varieties at 90°C. Meanwhile, significant mortality was detected at 80°C and 90°C also. On the other hand, *S. indicum* and *Cicer arietinum* did not show any significant effect on *C. elegans* and did not lead to effective mortality.

This type of cure has been used worldwide for thousands of years in Bohemia. They were used to throw henbane seeds on glowing coals or hot iron plates and let the patients inhale the evolving steam. Also, the narcotic and intoxicating effect of extract and vaping has been exploited in many ways. In Greek antiquity, the pagan priests used them before questioning the oracle (Auflage, 1970). Therefore, we suggest that the thermal release from *H. niger* and other medicinal plants might be used as alternative natural substances for antimicrobial activity of which also play a great role in biomedical applications and the discovery of new drugs.

5.5. Secondary Metabolite Profiling

5.5.1. Metabolic diversity of hairy root exudates of *Hyoscyamus niger* using HPLC-ELSD

A comparison of metabolic profiles of hairy root exudates collected in traditional shaken flasks and in the bioreactors revealed interesting differences. Results of HPLC-ELSD chromatograms revealed that certain metabolites accumulated only in the bioreactor but not in flask cultures and that the concentrations of most metabolites found in both cultivation methods were higher in hairy root exudates collected in bioreactors while only a few metabolites in flask cultures. Apart from confirming the suitability of the bioreactor for the production of hairy root exudates, these results revealed that phenotypic plasticity in root exudation is surprisingly large, encompassing quantitative differences. The results confirm earlier reports of a strong effect of cultivation conditions on the composition of hairy root exudates (Renouard *et al.*, 2018, Ludwig-Muller *et al.*, 2014, Palavalli *et al.*, 2012). Switching from shaken flasks to bioreactors affected relative concentrations of individual metabolites, but it also caused changes in the presence/absence of metabolites.

A comparison of the metabolic profiles of hairy root exudates obtained from three varieties of *H. niger* revealed quantitative differences. The similarity patterns revealed by nMDS clustering (Figures 69) for both cultivation systems and the Euclidean distances (Figures 70) among profiles obtained from flask cultures were congruent. nMDS clustered the varieties Iraq and Germany as most similar for both cultivation techniques. The metabolic profiles of the varieties Iran exhibited the highest variation among replicates and the largest distance from the other two varieties. Euclidean distances corroborated the close relationship between the varieties of Iraq and Germany for metabolic profiles of exudates obtained from shaken flasks only. According to metabolic profiles obtained from the bioreactors, varieties, Iraq, and Germany formed the second most similar pair. The likely reason for the inconsistency in the similarity ratings of the two methods was that nMDS clustering was based on the presence/absence of metabolic signals, while euclidean distances were calculated from normalized intensities. In other words, the relative contribution of differences in the intensities of shared metabolites in the metabolic profiles of the exudates obtained from bioreactors for the varieties Iraq and Germany was apparently larger than the relative contribution of the differences in shared metabolites to the euclidean distance between the varieties Iran and Iraq. The low 2D-stress value of nMDS indicated that the ordination in two dimensions was adequate; therefore, it cannot be suggested

that the difference in the ranking of similarity patterns between the two methods resulted from an artifact of data processing.

While chemical profiling grouped the varieties Iraq and Germany as the most similar, the genetic analysis grouped the varieties Iran and Germany as the most similar. The gene sequence used was short, but the sequence identity between the varieties Germany and Iran was compelling. It has been known that patterns of genetic and metabolic similarities are often incongruent (Laurentin *et al.*, 2008, Sarrou *et al.*, 2017, Houshyani *et al.*, 2012). As an explanation for this discrepancy in sesame it was suggested that the synthesis of secondary metabolites diversified very fast (Laurentin *et al.*, 2008), possibly because of selection pressure exerted on these metabolites due to their role in the protection of sesame plants from pathogens (Syed *et al.*, 2015). Other authors suggested that certain metabolic patterns are buffered against changes, which would result in a suppressed rather than accelerated diversification. In our opinion, addressing the relationship between genetic and metabolic diversity requires understanding the biological functions of the metabolites that constitute chemical profiles recorded by nontargeted chemical analysis. So far, most chemical signals recorded in metabolic profiles of non-model organisms such as *Hyoscyamus niger* cannot be even assigned to chemical identities. It needs a long way to understand the biological relevance of metabolic diversity, which is a prerequisite for the elucidation of the relationship between metabolic and genetic diversity.

5.5.2. Lignan profiling

Lignan contents of 25 sesame varieties were screened using High Performance Liquid Chromatography-mass spectrometry (HPLC-MS), and the levels of the lignans in the seeds are summarized in (Table 25).

Significant variation in Sesamin, Sesamolin, Sesaminol, Pinoresinol, and Sesamol contents in the seed of the 25 sesame varieties were observed. The results showed that the total lignan contents of these varieties ranged from 0.05 mg/g to 2.11 mg/g. The total lignan content recorded is higher than earlier records in the sesame lines of Tamil Nadu (Pathak et al., 2015). Significant variation was recorded between the different varieties of Sesame plant, this may relate to the environmental conditions since the varieties brought from different locations worldwide. Distribution of 25 sesame varieties based on their lignan content was clearly reflected the variability in biomedical activity and genetic variation. The distribution of this varieties somehow can represent the world-wide distributions (Figure 14, and 15) Furthermore, HPLS-MS analysis was carried out for three different plant tissue (hairy root, root, and seed)

which can be considered as the first study that investigates the lignan content of 25 sesame varieties within three different types of tissue.

The result of the current study demonstrates significant differences in lignan constitute between the hairy root and the normal root of sesame. The hairy root from shake flask culture shows higher lignan (sesamin) content comparing to the normal plant root. This means that the genetic modification of the hairy roots results in different gene integration and expression for compounds that are strongly related together. Such a system would offer new opportunities to examine the biochemical steps involved in regulatory work (Fu *et al.*, 2006).

This result indicates that the hairy root culture technique is a very good alternative technique for secondary metabolite production comparing to the intact plant. Recently, the bioreactors systems have been demonstrated to be one of the most important methods for the cultivation of hairy roots and the production of valuable plant-derived secondary metabolites (Yancheva *et al.*, 2019).

5.6. DNA Sequencing

5.6.1. Amplification of Specific Genes

Hyoscyamus niger is from Solanaceae or Nightshade family contains more than 3000 species covering a very large diversity. This species occurs worldwide and growing as large trees in rain forests to annual herbs (Aflitos *et al.*, 2014). Sesame is one of the oldest oilseed plants and is a part of the Pedaliaceae family. It is grown primarily in Asia, Africa, South America, in tropical, subtropical, and northern temperate countries of the globe (Uncu *et al.*, 2015).

In this study, we evaluated the genetic diversity of 7 *Hyoscyamus niger* and 25 Sesame varieties of worldwide distribution. Six specific primers, two genes were amplified from *Hyoscyamus niger*; *TR* 1, and *TR* 2 genes (designed by Ling Su, *Molecular Phytopathology and Mycotoxin Research, Göttingen University*) and two other genes were amplified from the *Sesamum indicum FAD2*, and *SAD* primers were amplified (Mondal *et al.*, 2015). Therefore, genetic diversity analysis based on four sequences were used to provide a reference for the conservation of genetic resources.

5.6.2. Alignment of the Sequences

The results of the multiple sequence alignment show that many variations were detected among the samples of *H. niger* when compared to the reference strain (Figures 75 and 76). An enormous difference was found in the *TR*1 gene among the varieties from Iraqi regions in

comparison to both varieties from Germany and Iran, which looks more compatible with the reference sequence. However, the amplified *TR*2 gene among the same varieties also showed variations among the varieties. However, the amplified genes in the *S. indicum* in different varieties showed less variation in comparison to *H. niger* genes. Interestingly, more similarities were observed among the isolates from different regions and precisely among those of Iraq.

5.6.3. Phylogenetic Analysis

The Phylogenetic tree declares an obvious grouping based on the varieties. The tree-based on the *TR*1 gene among the isolates exhibiting that samples from Iraq are grouped together with the high similarity between them while the constructed tree based on the *TR*2 gene showed both Iraqi samples of Hawraman and Penjwin are more similar to the Iranian samples and the reference sequence. Furthermore, both samples from Darai Mar and Hasanawa are similar in 92% of their sequences. The tree constructed based on both *FDA* and *SAD* genes of *Sesamum indicum* showed different groups, and the similarity among those varieties are quite interesting. For example, some of the Iraqi samples from Hawler and Ranya are more similar to the Korean sample, while others are more similar to Turkish or Syrian samples. However, the samples from Venezuela are quite different from most of the other isolates. Surprisingly, the constructed tree based on the *SAD* gene in *Sesamum indicum* showed a higher difference among the verities and formation of more groups.

The current results identified the characteristics of two sequences in *H. niger* and *S. indicum* and showed that the analysis of these regions represents a valuable technique for measuring genetic diversity. The analysis was relatively compatible with the biomedical applications and chemical profiling of these varieties.

5.6.4. Metabolic Diversity of Hairy Root Exudates and Genetic Diversity in *Hyoscyamus niger*

One of the goals of studies of chemical diversity in plants is to assess the relationship between metabolic and genetic diversity. The results of a limited number of studies indicated that patterns of genetic and metabolic similarity might be incongruent (Laurentin *et al.*, 2008, Sarrou *et al.*, 2017, Houshyani *et al.*, 2012). The bioreactor designed in this work will facilitate a comparison of root exudates and/or hairy root exudates for plant varieties with known genetic relationships. To demonstrate this approach on hairy root exudates of *Hyoscyamus niger* (Section 3.7.), we determined pairwise Euclidean distances among normalized metabolic profiles of hairy root exudates of three varieties of *H. niger*. To determine the effect of the

cultivation system on the composition of the exudates, the distances between metabolic profiles of each variety grown in bioreactors and in shaking flasks were determined.

The results showed that the differences among varieties were of the same magnitude as differences due to cultivation conditions. To obtain a first insight into the relationship between metabolic profiles and genetic distances among plant varieties from which the hairy roots were derived, the third intron of the *Tr2* gene was sequenced. Tr2 encodes tropinone reductase, which is involved in the synthesis of tropane alkaloids. The sequences were deposited in NCBI under Nos. MN017172 (varieties Iraq), MN017173 (varieties Iran), and MN017174 (varieties Germany). The number of nucleotides differences per 1000 bp between the sequences from the varieties Iraq/Iran and Iraq/Germany was 21 and 23, respectively, while the varieties Iran and Germany differed in only 2 nucleotides. Thus, the varieties of Iran and Germany appeared more closely related than each of them to the varieties of Iraq. The similarity between sequences of a *Tr2* gene of the varieties Iran and Germany was not reflected by the similarities of metabolic profiles of their hairy root exudates (Figure 67). Depending on cultivation conditions, the most similar profiles were Iraq/Germany and Iraq/Iran. The discrepancy between metabolic and genetic similarity is in line with the results of previous studies in other plant species (Laurentin *et al.*, 2008, Sarrou *et al.*, 2017, Houshyani *et al.*, 2012). In contrast, chemical profiles and microsatellite markers in *S. indicum* were correlated (Lamine *et al.*, 2017). *Hyoscyamus niger* is an attractive model to study the relationship between metabolic and genetic diversity in plants because it is a source of medicinally useful tropane alkaloids (Grynkiewicz and Gadzikowska, 2008). Attempts to produce tropane alkaloids in hairy root cultures of *Hyoscyamus niger* began decades ago (Yun *et al.*, 1992), yet nothing is known about the genetic variation within the species. The availability of small-scale, inexpensive bioreactors will allow for comprehensive sampling of metabolic diversity in hairy root cultures of *H. niger*, which will facilitate the selection of varieties for production purposes and enable studies of the relationship between metabolic and genetic diversity in this medical plant.

CONCLUSIONS

This study was carried out on seven varieties of Henbane and 25 varieties of Sesame using the most advanced protocols regarding hairy root culture, bioreactor, biomedical investigations, chemical profiling, and DNA sequencing. For the present study, the following conclusions can be made:

1. The aseptic protocol for seed germination using GA3 was the ideal procedure for sterilization and hairy root production, since the viability of the leaves remain optimal and hairy root initiation reaches the highest level, this decrease the time and cost and increase the yield of hairy roots.
2. The design of a simple bubble bioreactor which shows high hairy root biomass yield and good quality of hairy root and increases the secondary metabolite production, furthermore it is easy to use with a low rate of contamination.
3. The using of the *Caenorhabditis elegans* model for the toxicity examination is one of the best protocols. Moreover, *C. elegans* has a high reproductive capability and can proliferate even from a very small population and adequate sensitivity with high resistant to environmental stress. During this study, using *C. elegans* for antinematode activity through different plant specimen (root, hairy root, and seed) and different plant extracts (Essential oil, methanol extract, and flask oil) in one comparative study, gives an ideal view about the antinematode activity by *H. niger* and *S. indicum*.
4. Reviewing the previous literature, our study, for the first time, studied the antinematode activity of the thermal release from *H. niger*, and the highest mortality 100% were obtained in the standard condition in the newly designed instrument.
5. The lignan content of hairy root was larger than the normal root. Also, the secondary metabolite quantitatively and qualitatively was higher in bioreactor exudates than the flask culture except for Iraq varieties. This gives the vision for the ideal usage of this simple bioreactor in the production of important plant secondary metabolites.
6. Using DNA sequencing, which is the most advanced technology for all varieties of both plants *H. niger* and *S. indicium*, gives a clear idea about the relativeness and distance between these varieties and how this act on their secondary metabolite and biomedical activity.

RECOMMENDATIONS

This study is distinctive because it includes several research areas related to pharmacognosy and new drug discovery, hairy root culture, genetic diversity, and chemical profiling. In this manner, the following recommendations can be highlighted:

1. The chemical profiling of (Seed, Root, and Hairy Root) samples of *Hyoscyamus niger* should be carried out regarding the Lignans and alkaloid content. This will provide the whole view of comparison and leads to discovering new Lignan compounds.
2. Creating a new system for biomedical analysis requires further study regarding thermal release protocol by screening more medicinal plans. Furthermore, detailed chemical analysis is required for the thermal release to distinguish and detect the exact active compound that is actively released and show biomedical activity; this may open a new window in pharmacological studies.
3. Further studies should be carried out to validate the anthelmintic effects, insecticidal, and anti-microbial activity of *Hyoscyamus niger* and *Sesamum indicum.*
4. A more detailed study should be cared out to examine the alkaloid constitutes of *Hyoscyamus niger,* root, hairy root, and seed. Because previous studies regarding *H. niger* alkaloids (Hyoscyamine, Hyoscyamal, Hyoscyamoside A &B, Hyoscyamilactol, Littorine, Hyoscine, Atropine, and Scopolamine) in the root, hairy root and seed were not been conducted yet.
5. Further study is needed on the secondary metabolite in hairy root exudate of the medicinal plant.

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6. APPENDICES

6.1. Appendix A: Published Articles

applied sciences

MDPI

Article

Small-Scale Bioreactor for Sterile Hydroponics and Hairy Roots: Metabolic Diversity and Salicylic Acid Exudation by Hairy Roots of *Hyoscyamus niger*

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Abstract: The volume and complexity of commercial bioreactors for sterile hydroponics and hairy roots are too large for comparative analysis of many cultures. Here a small-scale bioreactor fabricated from standard glass materials and suitable for both airlift and bubble aeration mode is described. The performance of the bioreactor was tested by growing oilseed rape (*Brassica napus* L.) and rose plants (*Rosa canina* L.) in sterile hydroponics and by cultivating hairy roots of henbane (*Hyoscyamus niger* L.) and sesame (*Hyoscyamus niger* L.). Plants grown in hydroponics for up to six weeks did not show chloroses or necroses. Hairy roots grew faster or comparably fast in bioreactors as compared to shaking flasks. Root exudates of roses and exudates of hairy roots of henbane were subjected to targeted and nontargeted analysis by HPLC coupled with optical and mass spectrometric detectors. The diversity and concentration of hairy root exudates were higher in bioreactors than in shaking flasks. The composition of hairy root exudates of three accessions of *H. niger* did not match the genetic relatedness among the accessions. Hairy roots of *Hyoscyamus niger* exuded salicylic acid in amounts varying among plant accessions and between bioreactors and shaking flask cultures.

Keywords: hairy roots; root exudates; bioreactor; salicylic acid; *Hyoscyamus niger*; metabolic diversity

1. Introduction

Roots of land plants exude small molecules, oligosaccharides, and proteins into the soil. The secreted compounds called root exudates serve multiple functions: they facilitate mobilization of soil nutrients [1–3], modulate the composition of microbial communities of the rhizosphere [4], and attract or repel soil micro- and mesofauna, affecting crop health and productivity [5]. Exudation of metabolites by roots is modulated by nutrient availability [6] and herbivore infestation [7]. Root exudates in turn serve as chemical signals for symbiotic and pathogenic microorganisms [8–10], soil herbivores and their predators [11].

Hairy roots are plant roots genetically transformed with the help of *Agrobacterium rhizogenes* that exhibit neoplastic growth and can be maintained indefinitely in sterile media without externally added phytohormones [12,13]. Because hairy root cultures consist entirely of differentiated roots and can be easily cloned, they are an ideal model for studies of root biochemistry [14,15] and root-specific processes such as nodulation [16] and the synthesis of root-specific metabolites [17]. Hairy roots also proved useful in studies of interactions between crop plants and herbivores [18]. Biomass production in hairy root cultures is higher than in callus or suspension cultures, therefore hairy roots have been exploited for the production of plant secondary metabolites [12,19–22]. This strategy appears

particularly promising for large-scale production of metabolites that are primarily synthesized in the roots, such as certain tropane alkaloids of medical interest [23] and glucosinolates with chemoprotective potential [24]. Optimization of the activity of desired biosynthetic pathways can further increase the efficiency of secondary metabolite production by hairy roots [25–28]. Recently genetic engineering of hairy roots received a boost by an adaptation of CRISPR/Cas9 technology for delivery by *A. rhizogenes*, which combines genome editing and hairy root induction into a single step [29]. Root exudates of hydroponically grown genetically engineered plants [30] as well as hairy roots of genetically modified plants [31,32] can also be used for the production of proteins for diagnostic and medical purposes such as antibodies, antigens for immunization, and growth factors. Optimization of the production system requires comparing multiple genetically enhanced lines because the efficiency of promoters used to drive heterologous gene expression varies among transformation events and even among different parts of the same plant [33]. Similarly, the expression of target genes and the yield of the desired products varies among hairy root lines [21,28]. Production of natural products by plants that have not been genetically engineered requires testing multiple accessions, too, because secondary metabolite synthesis varies among plant accessions in a way that is often not predictable based on the genotype [34,35]. Furthermore, cultivation conditions strongly affect production of plant secondary metabolites in hydroponics [36] as well as in hairy root cultures [21,37]. For these reasons, multiple cultures have to be grown in parallel during the optimization of hairy root-based production systems.

A major challenge for hairy root cultures is the supply of oxygen [38]. Different concepts have been pursued to improve oxygen supply to hairy roots in mid- and large-scale bioreactors [22,39]. Apart from conventional stirred bioreactors [40] and stirred bioreactors modified to reduce sharing damage [22,41], hairy root cultures have been grown in plastic sleeve reactors [42], bubble reactors [43], mist reactors [44,45], airlift reactors [46], and liquid-dispersed reactors [47]. The implementation of these concepts focused on midscale bioreactors for research on hairy roots and on industrial bioreactors. Micro and nano bubble generators have recently been introduced into bioreactor design [48], inspired by technologies for the delivery of oxygen and ozone in wastewater treatment [49]. The large surface of micro/nano bubbles as compared to conventional bubbles improves oxygen transfer efficiency. The drawback of micro and nano bubble generators is that they require high-pressure pumps. Furthermore, in airlift mode the liquid velocity decreases with bubble radius for bubbles smaller than 100 μm [50]. Because micro bubbles are smaller than 50 μm and nano bubbles are smaller than 200 nm [49], micro bubbles and especially nano bubbles are less suitable to drive liquid transfer in airlift bioreactors.

Small-scale inexpensive bioreactors for screening purposes and comparative studies are lacking, limiting our ability to address differences in the exudation potential of hairy root cultures derived from different plant accessions. Studies of the relationship between genetic polymorphism and secondary metabolite accumulation in plant tissues e.g., [34,51,52] and in root exudates [53] calls for comparative analysis of numerous plant accessions. Exclusion of the effect of bacteria and fungi colonizing plants as endophytes, pathogens, and inhabitants of the phyllosphere require axenic conditions, which can most conveniently be achieved in sterile hydroponic cultures. Lack of small and economical incubators for sterile hydroponics limits these studies.

Our laboratory studies the chemical diversity in sesame [34], extending the focus from seeds to roots and hairy roots and their exudates. In another project we study biological functions of salicylic acid, which may fulfill different roles in different species and even in different tissues of the same plant species. Salicylic acid is known to act as a signal of plant defense response in plant shoots [54] but we also found it in xylem sap of plants infected with a fungal pathogen [55] and in root exudates of healthy plants, where it facilitated mobilization of soil phosphorus [1]. Salicylic acid was also reported to participate on shaping microbial communities of the rhizosphere [56], serving as a signal in interorganismal interactions. Comparative analysis of salicylic acid exudation by different plant accessions requires growing multiple sterile cultures in parallel. In another project in our laboratory we study replant disease of roses. This project relies on the analysis of root exudates because we pursue a

hypothesis explaining the disease by the effects of certain components of root exudates accumulating in the rhizosphere of roses [57]. Root exudates of rose accessions known to suffer from replant disease have to be collected under sterile conditions, their components identified and the phytotoxicity of these components determined. Progress in all three projects has been hampered by the lack of small bioreactors for sterile hydroponics and hairy root cultures.

The objective of this work was to develop a small-volume, low-cost bioreactor and validate its suitability for hydroponic cultures of *Brassica napus* and *Rosa canina*, for growing hairy roots of *Hyoscyamus niger* and *Sesamum indicum* and for the collection of root exudates and hairy root exudates suitable for HPLC analysis.

2. Materials and Methods

2.1. Bioreactor Design

The concept and operating principle of the bioreactor are shown on Figure 1. The body of the bioreactor is connected to an external draft tube. In airlift aeration mode, air bubbles released from a glass frit at the bottom of the draft tube saturate medium with oxygen and drive nutrient medium flow between the draft tube and the reactor body (Figure 1A). The aeration mode can be switched from airlift to bubble mode by attaching air supply to the port on the top of the draft tube and closing the port at the bottom of the draft tube (Figure 1A). The facility to control the aeration mode by connecting air supply to an appropriate port of the draft tube with remaining parts of the reactor unchanged is a distinctive feature of the design.

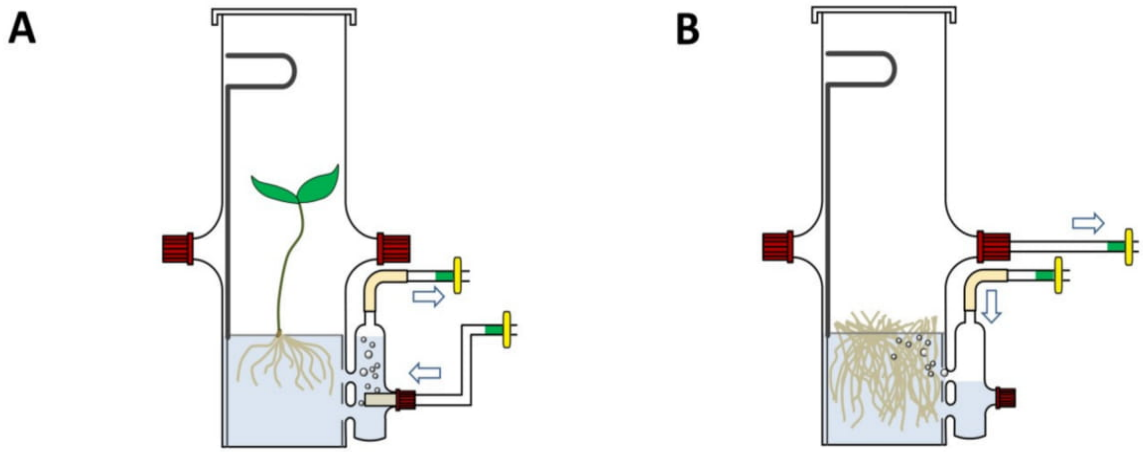

Figure 1. Scheme of bioreactor operating in airlift mode (**A**) and bubble aeration mode (**B**).

The second part of the reactor is an internal cylinder inserted into the reactor body. The internal cylinder holds a supporting disk that carries plants or hairy roots. The disk, which loosely resides on the perimeter of the cylinder (Figure 2A, part 1), can be cut from a perforated plate or mesh made of plastic, stainless steel or other materials. The internal cylinder is equipped with a handle for easy removal to facilitate harvesting cultivated material under a sterile bench.

All components and working principles of the bioreactor have been known but the double function of the external draft tube and the way how the bioreactor is assembled from common glass components (see also the following section) are new to this design.

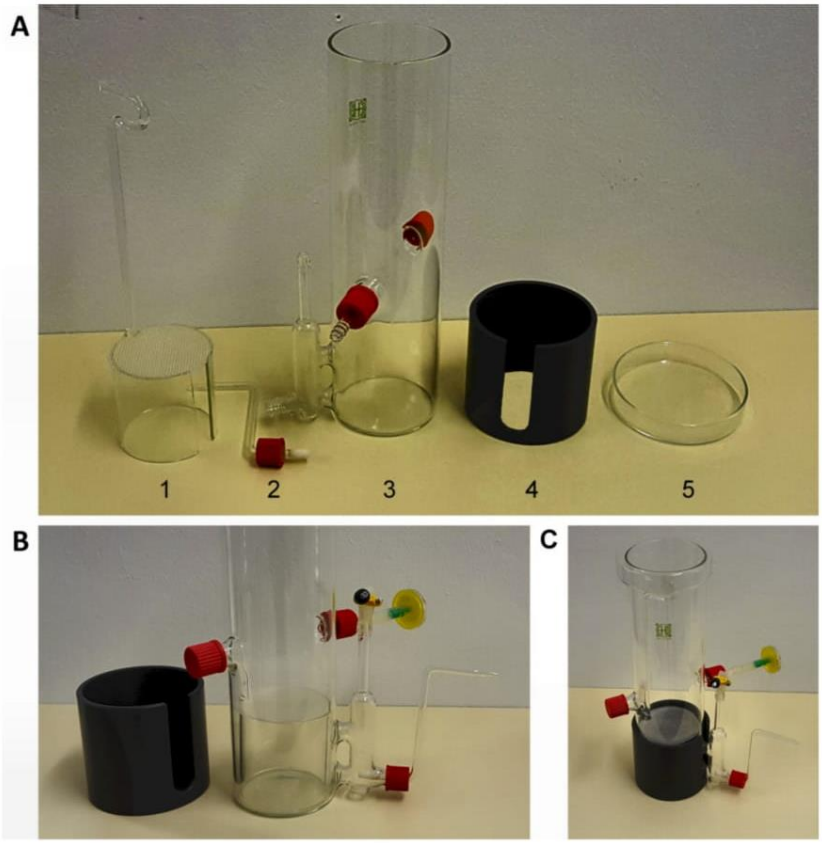

Figure 2. Bioreactor for sterile hydroponics and hairy root cultures. (**A**) Components of the bioreactor are (1) internal cylinder with a polypropylene mesh, (2) connector for air supply with a glass frit, (3) reactor body with an attached draft tube, (4) light-shielding external cylinder made of polyvinyl chloride, and (5) Petri dish serving as a lid. (**B**) Detailed view of a bioreactor fitted with an air supply tube. (**C**) Full view of an assembled bioreactor.

2.2. Fabrication of the Bioreactor

The components of the bioreactor are shown in Figure 2A. The reactor body with an attached draft tube (Figure 1A, part 3) and an internal glass cylinder (Figure 1A, part 1) were made of glass tubes. The light-shielding external cylinder (Figure 1A, part 4) was cut from a polyvinyl chloride tube. The body of the reactor had an external diameter of 90 mm, a height of 270 mm and a wall thickness of 2.5 mm. The draft tube of 18 mm diameter and 75 mm height was connected with the reactor body at two sites via glass tubes as shown in Figure 2A, part 3 and Figure 2B. It was furnished with two connectors: a bottom connector accommodated a glass tube with a frit supplying air in airlift operation mode (Figure 2A, part 2). The top connector served as air supply in bubble aeration mode. The body of the reactor was equipped with two ports for the collection of volatiles, for an optional CO_2 supply or for air exhaust in bubble aeration mode. Ports that were not in use were closed with screw cups. The internal cylinder had a diameter of 84 mm and a height of 70 mm. The cylinder held a polypropylene mesh, which rested on its perimeter as a support for plants or hairy roots during cultivation (Figure 2A, part 1). A handle attached to the internal cylinder was made of a glass rod of a diameter of 5 mm. A clearance of 20 mm width was cut into the internal cylinder (Figure 2A, part 1) at a position facing the draft tube (Figure 1A,B) to allow exchange of growth medium between the body of the reactor and the draft tube and to facilitate air supply in bubble aeration mode. A glass Petri dish with an internal diameter of 91–92 mm served as a lid (Figure 2A, part 5).

2.3. Operation of the Bioreactor

For sterile hydroponics, the reactor was filled with nutrient solution just to the level of the mesh supporting the plants, which required about 450 mL medium per reactor. The reactor was operated in airlift mode with the upper port of the draft tube closed with a filter and the air supply attached to the lower port of the draft tube. The air flow was 480 mL/min.

For growing hairy roots, the reactor was steam-sterilized and filled with nutrient solution to the level of the supporting mesh or above, depending on whether aerial growth was desirable. Hairy root cultures are known to suffer from inhomogeneous growth and inferior yields when grown in air-lift reactors [46], therefore bubble aeration mode was used. The air flow was 670 mL/min. In some experiments, the lid was loosely place over the reactor, both ports of the body of the reactor were closed with screw cups to and the flow of air leaving the reactor below the led prevented contamination. In other experiments, the lids were sealed in place with Parafilm and one of the ports of the reactor body serving as an air exhaust was equipped with a hydrophobic sterile filter (Midisart, Sartorius, Göttingen, Germany).

Low-cost aquarium pumps connected to the reactors via Midisart filters were used as air supply. The reactors were operated in an environmental chamber (Mytron, Bio-u. Solartechnik GmbH, Heiligenstadt, Germany) set to a constant temperature (varying among the experiments as described in Section 3) and 12 h light per day. We have not measured oxygen concentration in growth medium because the concentration within in the bulk of hairy roots is likely to vary with the location of the probe and accurate methods for the determination of volumetric mass transfer coefficient, such as a rapid switch between input gases with different oxygen concentrations or the dynamic pressure method [58], were beyond the scope of this work.

2.4. Plant Material

Seeds of *Brassica napus* var. *napus*, rapid cycling accession were provided by P.H. Williams (Crucifer Genetics Cooperative at the University of Wisconsin-Madison, WI, USA). Sterile cuttings of rose plants (*Rosa canina* L., variety "The Fairy") were purchased from Institut für Pflanzenkultur (Schnega, Germany). Accessions of *Hyoscymus niger* and *Sesamum indicum* used in this study are listed in Tables 1 and 2.

Table 1. Hyoscyamus niger.

Label	Origin	Year	Source
Iraq	Iraq, Daray Mar	2014	Collected by ZJK
Iran	Iran, Isfahan	2015	Pakan Bazr *
Germany	Germany, Göttingen	2016	Botanical garden

* Seeds were provided by Pakan Bazr company, Iran.

Table 2. *Sesamum indicum* accessions.

Label	Origin	Year *	Accession
India	India, Hyderabad	2004	Acc. 92–2918 **
Japan	Japan	2004	Acc. 92–3030 **
Sumer S2b	Iraq, Erbil	2014	Sumer S2b ***

* Year of the acquisition of seeds from the collection. ** Centro Nacional Inv. Agropecuarias (CENIAP) Germplasm Bank, Venezuela. *** Ministry of Agriculture, Regional Government of Iraqi Kurdistan, General Directorate of Research, Extension and Training.

2.5. Analysis of Root Exudates by HPLC-DAD

Growth medium was filtered through paper filter to remove root fragments and concentrated in a rotary evaporator to 1/4 volume. The concentrate was extracted with equal volume of ethyl acetate, the watery phase was acidified with 1% acetic acid and extracted with a new portion of ethyl acetate.

The extracts were combined and solvent was removed in vacuum. Three biological replicates were prepared and extracts of culture medium incubated in bioreactors under the same conditions yet without plants were used as blanks. Thirty-three milligrams of dried residue were suspended in 1.5 mL of methanol/water (1:1) and sonicated for 5 min. The suspension was filtered through 0.4 µm filters and 10 µl were injected into HPLC system 1290 Infinity II with a DAD detector G7117B (Agilent, Darmstadt, Germany) equipped with a Varian Polaris C18 Ether column (3 µm particle size, 2 × 100 mm) kept at 35 °C. LC-MS grade methanol and distilled water were acidified with 0.1% formic acid and used for gradient elution from 95% to 0% water in 20 min at a flow rate of 0.2 mL/min. Light absorption spectra were recorded from 220 to 550 nm and chromatograms at the wavelengths 229, 254, 280, and 320 nm were monitored. The bandwidth was set to 4 nm and the reference wavelength to 600 nm.

2.6. Induction of Hairy Roots

Agrobacterium rhizogenes ATCC 15834 was grown in YEB medium [59] (0.5% peptone, 0.1% yeast extract, 0.5% meat extract, 0.5% sucrose, 0.049% $MgSO_4$. $7H_2O$, 1.5% agar, pH 6.8–7.2), at 25–26 °C for 36 h in the dark on a rotatory shaker (100 rpm). The cells were harvested by centrifugation at 5000× *g* for 15 min, re-suspended in MS medium (Murashige and Skoog medium, [60]) and set to the OD_{600} of 1.3. Leaves were harvested from four weeks old plants grown under sterile conditions from superficially sterilized seeds, cut into squares of 1 × 1 cm, placed on 1/2 MS medium solidified with 0.7% agar and co-cultivated with *A. rhizogenes* for 30 min at room temperature. After the incubation, leaf segments were dried with sterile filter paper and placed onto phytohormone-free MS medium supplemented with 2.2% (w/v) sucrose and solidified with 0.7% agar and incubated in darkness at 22 °C. After 72 h the segments were transferred onto fresh medium supplemented with cefotaxime at 500 mg/L. About two weeks after the inoculation the formation of the first roots was observed. One root was excised from each leaf segment. These roots were treated as hairy root clones. The roots were subcultured each 30 days. After six passages, sterile hairy roots were transferred to antibiotic-free medium.

2.7. Analysis of Hairy Root Exudates by HPLC-ELSD

Three biological replicates were prepared and culture medium was used as a blank. The samples were analyzed by HPLC using Agilent 1290 Infinity II system coupled to an Agilent 1260 evaporative light-scattering detector (ELSD) (Agilent, Darmstadt, Germany). For the ELSD, the evaporation temperature was set to 42 °C, the nebulizer temperature to 40 °C and the nebulizer N_2 flow amounted 1.6 L/min. A Varian Polaris C18 Ether column (3 µm particle size, 2 × 100 mm) was used for separation. The column temperature was kept at 35 °C and samples of 40 µL were injected. LC-MS grade methanol obtained from Chemsolute (Th. Geyer, Renningen, Germany) and distilled water purified by Arium Pro water purification system (Sartorius, Göttingen, Germany) were acidified with 0.1% LC-MS grade formic acid (Honeywell Fluka, Bucharest, Romania) and used for gradient elution from 90% to 2% water in 15 min at a flow rate of 0.2 mL/min.

2.8. Analysis of Salicylic Acid Content in Hairy Root Exudates by HPLC-MS

The samples were analyzed by HPLC using an Agilent 1290 Infinity II system (see Section 2.7 for details) with the column maintained at 35 °C. LC-MS grade methanol and distilled water were acidified with 0.1% formic acid and used for gradient elution from 95% to 0% water in 20 min at a flow rate of 0.2 mL/min. The HPLC system was coupled to an Agilent 6545 QTOF equipped with a Jet Stream source operated with the following settings: drying gas temperature 320 °C, drying gas flow 8 L/min, nebulizer pressure 35 psig, sheath gas temperature 350 °C, and sheath gas flow 11 L/min. Negative mode data were acquired with three spectra pre second recorded from 100 to 1700 m/z. External calibration was prepared from salicylic acid solutions in 50% methanol.

2.9. Primer Design and Sequence Analysis

The sequence of Tr2 gene from *Hyoscyamus niger* (accession No. AB026545.1, tropinone reductase II) was used for the design of primers for PCR and sequencing. The primers (forward primer TCTGA ACGAG AAGAG TTTAT GAAGA and reverse primer AAGTG ATAAG CAGCC TCAAA GT) amplified a segment of the gene from 1543 nt to 2237 nt, producing an amplicon of 695 bp. PCR products were sequenced by Sanger method from both ends (Macrogen Europe, Amsterdam, The Netherlands), the complementary reads were aligned and ambiguities were resolved by inspection of the electropherograms. An alignment of the sequences from 1592 nt to 2165 nt (original numbering of AB026545.1) was constructed using Clustal W implemented in MEGA 10.0.4 [61] and used to calculate nucleotide differences per 1000 bp.

3. Application Examples

The performance of the bioreactor was tested by growing two plant species in sterile hydroponics and collecting root exudates; by comparing growths rate of hairy roots generated from two plant species; and by collecting and analyzing hairy root exudates.

*3.1. Growing Oilseed Rape (*Brassica napus *L.) in Sterile Hydroponics*

Ten surface-sterilized seeds of a rapid cycling variety of *Brassica napus* were placed into bioreactors filled with half-concentrated MS medium [60] without sucrose and operated in airlift mode. The plants grown at 24 °C for three weeks have not shown any chloroses or other negative effects of cultivation in restricted space and at high air humidity [62,63] (Figure 3).

Figure 3. Oilseed rape (*Brassica napus* L.) grown in sterile hydroponics. Ten plants per bioreactor were grown in 0.5× MS medium for three weeks at 24 °C with 12 h light per day. External light-shielding cylinders were removed for the photography.

*3.2. Growing Rose Plants (*Rosa canina *L.) in Sterile Hydroponics*

Rose cuttings were adapted to light for 3 days at 20 °C under long day conditions before transfer to bioreactors. A single rose plant per bioreactor was cultivated for six weeks in half-concentrated MS

medium with 2 g sucrose per liter and pH-value set to 5.8 with KOH in bioreactors in the airlift mode (Figure 4). The plants were grown at 20 °C for four weeks.

All roots formed on cuttings were adventitious by definition, yet strong white roots penetrating the plastic mesh and growing downwards resembled primary and seminal roots, while dark roots growing on the surface of the mesh and supporting the plants in a vertical position resembled crown roots (Figure 4). The roots growing downwards in medium remained white for the entire cultivation period without signs of necroses, which typically occur in medium solidified with agar (A. Sirrenberg, unpublished observation) due to replant disease syndrome [57].

Figure 4. Rose plant (*Rosa canina* L.) growing in sterile hydroponics in 0.5× Murashige–Skoog medium supplemented with 2% sucrose in a bioreactor operated in airlift mode.

3.3. Collection and Analysis of Root Exudates of Rose (Rosa canina L.) by HPLC-DAD

Rose plants (*Rosa canina* L.) were grown in medium with 2% sucrose for the collection of root exudates as shown in Figure 4. After four weeks, plants were removed and medium was filtered and stored at −20°C. For the chemical analysis of root exudates, exclusion of microbial contamination is crucial because microorganisms use the exudates as nutrients and contaminate the medium with their own metabolites. In 11 experiments with 3 to 6 parallel cultures each, contamination occurred in less than 5% cultures. A total of 13.2 L medium were collected, concentrated in vacuum and extracted with ethyl acetate (see Section 2.5.) for the analysis and purification of selected components of root exudates. Figure 5 shows a typical result of the analysis of root exudates of rose plants collected in bioreactors by HPLC coupled to a UV detector (HPLC-DAD).

3.4. Growing Hairy Roots of Hyoscyamus niger and Sesamum indicum

To compare the yield of hairy roots in the bioreactor and in shaken flask cultures, hairy roots originating from three accession of each *Hyoscyamus niger* and *Sesamum indicum* were cultivated in bioreactors and in shaking flasks. The cultures were initiated by 100 mg of hairy roots cut into 1 cm-long pieces. Culture flasks of 300 mL volume contained 50 mL MS medium supplemented with 2.5% sucrose. The cultures were grown on a shaker at 90 rpm in the dark at 25 °C and in bioreactors operated in bubble mode with an air flow of 670 mL/min (Figure 6).

After 30 days, hairy roots were harvested by filtration, blotted dry with sterilized filter paper, frozen at −20 °C overnight and lyophilized for 72 h. Then the dry weight of hairy roots was determined (Figure 7).

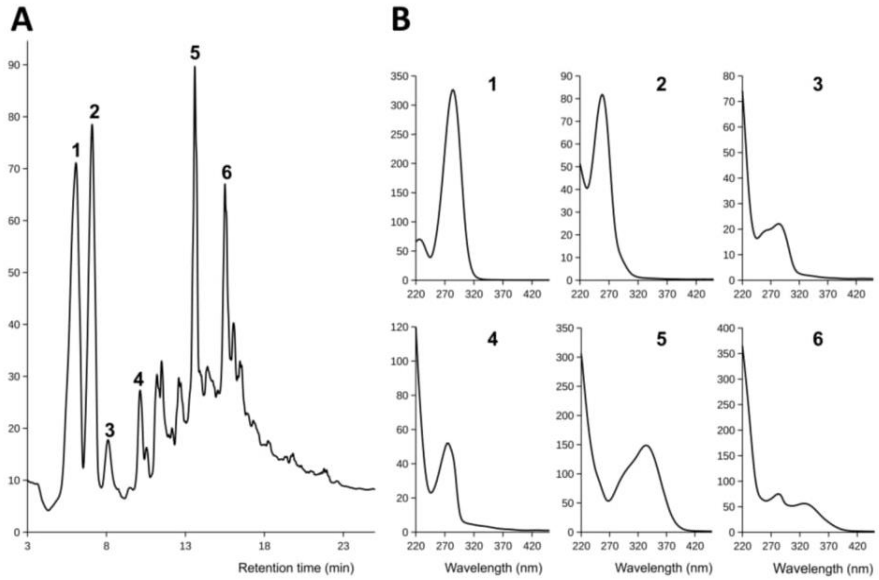

Figure 5. HPLC analysis of root exudates of rose (*Rosa canina* L.) collected in the bioreactor. (**A**) HPLC chromatogram of root exudates separated on a reverse-phase column, eluted with a methanol gradient and detected by light absorption at 254 nm. (**B**) UV absorption spectra of major phenolic compounds recorded using a diode array detector coupled to HPLC.

Figure 6. Hairy root cultures generated from several accessions of *Hyoscyamus niger* in bioreactors operated in a bubble aeration mode.

The biomass of hairy roots varied among accessions but we have not investigated these differences statistically because our purpose was to compare the performance of the bioreactor with that of flask cultures. In *Sesamum indicum*, the biomass of hairy roots grown in bioreactors was larger than or similar to the biomass harvested from shaking flasks. In *Hyoscyamus niger*, more biomass was harvested from bioreactors than from flasks in all three plant accessions studied (Figure 7). We assume that better supply of oxygen in the bioreactor was the cause of these differences.

3.5. Collection and Nontargeted Analysis of Hairy Root Exudates of Sesamum indicum

Growth medium of hairy root cultures grown as described above was cleared by centrifugation for 10 min at 2900× *g* to remove root fragments. The medium was used for HPLC-ELSD analysis

without cleanup. Figure 8 shows a comparison of HPLC-ELSD chromatograms of hairy root exudates obtained from the bioreactor and from a flask culture for one accession of sesame.

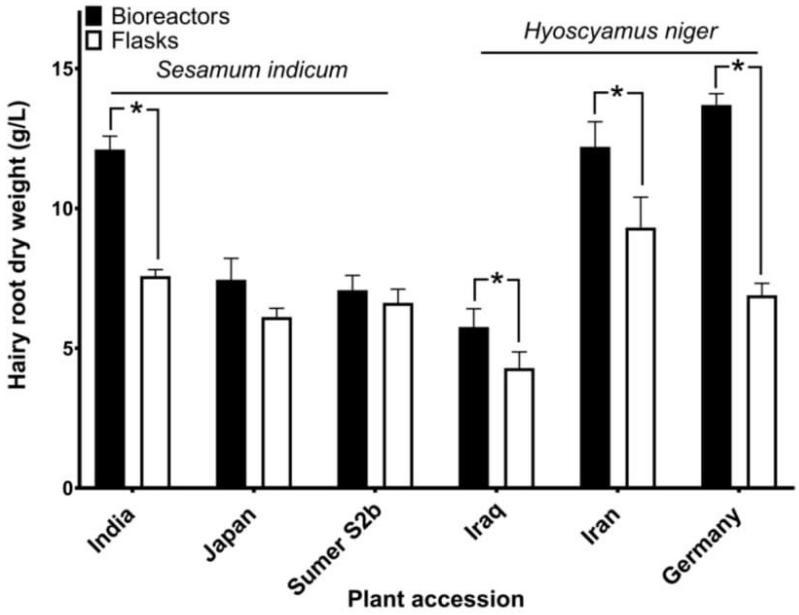

Figure 7. Biomass of hairy roots grown in bioreactors and flask cultures. Hairy root cultures originating from three accessions of each plant species were grown for 30 days and the dry weight of harvested biomass was determined. Asterisks denote significant differences between means (two-tailed *t*-test, n = 3, $p < 0.05$).

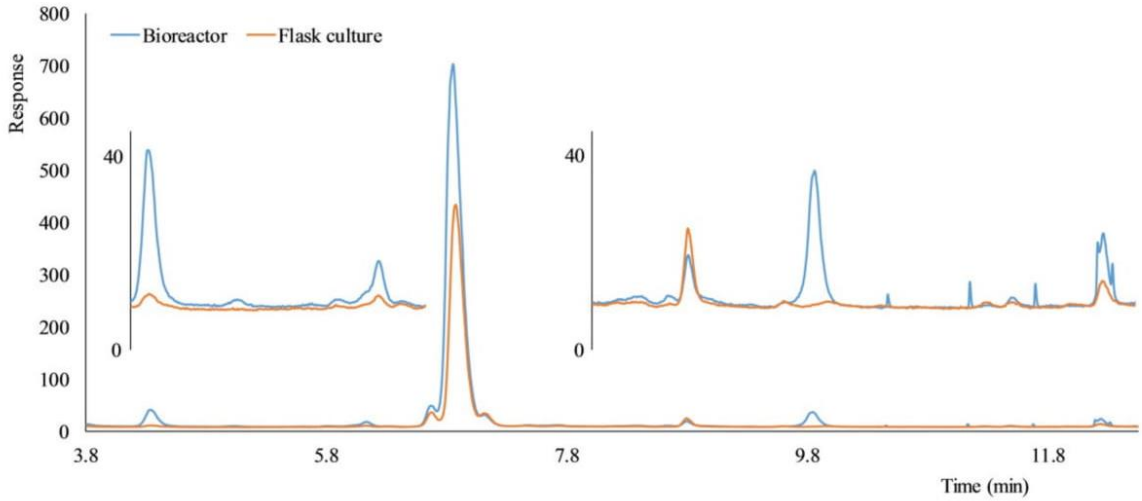

Figure 8. Comparison of HPLC-ELSD chromatograms of hairy roots exudates of *Sesamum indicum* (accession Japan) grown in the bioreactor and a flask culture. Culture fluid clarified by centrifugation was directly analyzed by HPLC-ELSD.

The amount and diversity of hairy root exudates obtained from a bioreactor were higher than the amount and diversity of exudates obtained from a shaking flask culture (Figure 8). Better oxygen supply in the bioreactors was the likely reason. Most importantly, all metabolic signals recorded in exudates from the flask culture were also found in exudates from the bioreactor, indicating that that few (if any) metabolites will be lost by replacing culture flasks with bioreactors.

3.6. *Analysis of Salicylic Acid Exudation by Hairy Roots of* Hyoscyamus niger

To test the suitability of the bioreactor for comparative studies of salicylic acid exudation by hairy roots, hairy root cultures generated from three accessions of *Hyoscyamus niger* and *Sesamum indicum* were grown in 0.5× MS medium with 2.5% sucrose in bioreactors operated in bubble mode and in shaken flasks. After 30 days, growth medium was collected, extracted with ethyl acetate and the extracts were analyzed by HPLC-MS. The results are shown in Figure 9.

Hairy roots of *Hyoscyamus niger* exuded salicylic acid into growth medium in amounts varying from 2 µg/L to 60 µg/L, while hairy root exudates of *Sesamum indicum* has not secreted detectable amounts of salicylic acid (data not shown). Cultivation conditions strongly affected the concentration of salicylic acid accumulating in nutrient solution. Hairy roots of one plant accession secreted larger amounts of salicylic acid in bioreactors, while hairy roots on another accession secreted much large amounts of salicylic acid in shaking flasks (Figure 9). This surprising result indicates that the response of salicylic acid pathway to environmental cues varies among populations of the same plant species. We speculate that these differences reflect selection pressures exerted by different environments on the plants. The result corroborates our assumption that studying differences in salicylic acid production among plants with different life histories may shed light into biological functions of this multifaceted metabolite.

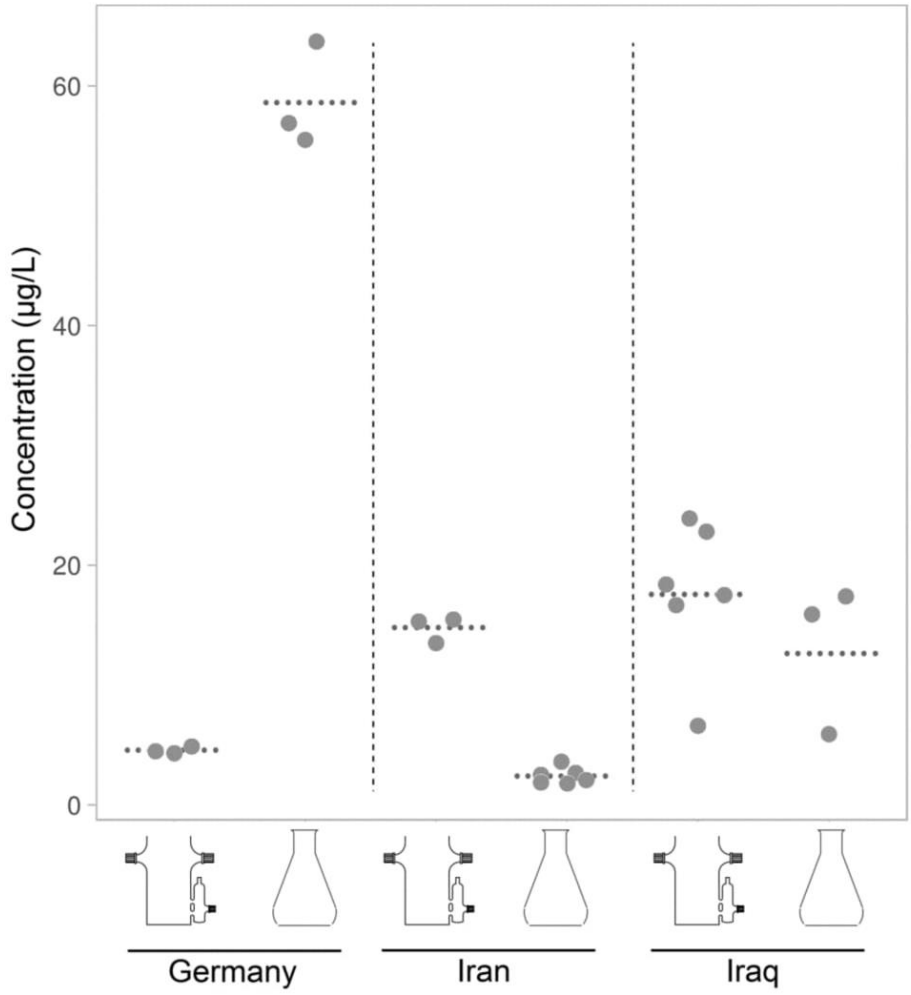

Figure 9. Salicylic acid accumulated in nutrient solution of hairy root cultures derived from three accessions of *Hyoscyamus niger* (designated Iraq, Iran and Germany) and grown in bioreactors and in shaking flasks for three weeks. Dotted lines show the means.

As far as we are aware, secretion of salicylic acid by hairy roots has not been reported yet. Adding salicylic acid to culture medium of hairy roots was used to stimulate the synthesis of alkaloids [64], including hyoscyamine and further tropane alkaloids [65,66], which are also produced by *Hyoscyamus niger*. The interplay between the secretion of salicylic acid and stimulation of alkaloid synthesis by externally added salicylic acid adds to the complexity of biological roles and effects of salicylic acid in plants.

3.7. Nontargeted Analysis of Hairy Root Exudates in Hyoscyamus niger: Comparison to Shaking Flasks

Hairy root cultures of *H. niger* were grown in the bioreactor operated in a bubble mode and in traditional shaken flask cultures. After 30 days, growth media were collected, extracted with ethyl acetate and the extracts were analyzed by HPLC. Because the components of the exudates were unknown, an evaporative light scattering detector (ELSD) was used, which allows for an approximate comparison of concentrations without authentic standards because the response factor of the detector is largely independent of the chemical structure of the analyte [67].

The results of the analysis of hairy root exudates by HPLC-ELSD are shown in Table 3. The average standard error of the intensity of HPLC signals in exudates obtained from bioreactors was 20%, compared to the average standard error of 28% for exudates from shaken flasks. Thus the reproducibility of the composition of hairy root exudates collected in bioreactors appears higher than the reproducibility of exudates collected in shaking flasks. The total intensity of HPLC-ELSD signals detected in exudates from bioreactors (average total per accession of 9024) was 1.6-times larger than the total intensity of signals from shaking flasks (average total per accession of 5692), indicating that hairy roots exuded larger amounts of metabolites in bioreactors as compared to shaking flasks.

Table 3. Exudates of hairy roots of *Hyoscyamus niger* collected in bioreactor and flask cultures. The exudates were analyzed by HPLC-ELSD. Only metabolites present in all three replicates were recorded.

Accession / Metabolite	RT *	Iran		Germany		Iraq	
		Bioreactor	Flasks	Bioreactor	Flasks	Bioreactor	Flasks
Compound **1**	3.6	N.d.	224 ± 191 **	N.d.	N.d.	N.d.	N.d.
Compound **2**	4.3	41 ± 4	N.d.	41 ± 4	N.d.	N.d.	100 ± 125
Compound **3**	6.1	128 ± 18	20 ± 2	24 ± 1	32 ± 2	30 ± 4	41 ± 2
Compound **4**	6.8	12,500 ± 600	2300 ± 00	5700 ± 490	5100 ± 200	7000 ± 300	5900 ± 200
Compound **5**	7.7	29 ± 5	N.d.	N.d.	N.d.	N.d.	N.d.
Compound **9**	9.5	40 ± 8	N.d.	N.d.	N.d.	35 ± 9	N.d.
Compound **10**	9.7	75 ± 29	750 ± 350	1300 ± 650	485 ± 175	N.d.	1750 ± 490
Compound **11**	9.9	42 ± 29	N.d.	N.d.	N.d.	N.d.	N.d.
Compound **12**	10.5	N.d.	N.d.	32 ± 2	52 ± 13	N.d.	120 ± 30
Compound **13**	11.1	N.d.	N.d.	51 ± 18	110 ± 41	N.d.	67 ± 2
Compound **14**	11.3	N.d.	N.d.	21 ± 5	N.d.	N.d.	N.d.
Compound **17**	14.8	N.d.	87 ± 8	N.d.	N.d.	N.d.	N.d.

* Retention time [min]. ** Signal intensity [peak area] as mean ± s.d.

3.8. Metabolic Diversity of Hairy Root Exudates and Genetic Diversity in Hyoscyamus niger

One of the goals of studies of chemical diversity in plants is to assess the relationship between metabolic and genetic diversity. The results of a limited number of studies indicated that patterns of genetic and metabolic similarity may be incongruent [34,35,68]. The bioreactor designed in this work will facilitate comparison of root exudates and/or hairy root exudates for plant accessions with known genetic relationships. To demonstrate this approach on hairy root exudates of *Hyoscyamus niger* (see Section 3.7.), we determined pairwise Euclidean distances among normalized metabolic profiles of hairy root exudates of three varieties of *H. niger*. To determine the effect of cultivation system on the composition of the exudates, we also determined the distances between metabolic profiles of each variety grown in bioreactors and in shaking flasks.

The results showed that the differences among accessions were of the same magnitude as differences due to cultivation conditions (Figure 10). To obtain a first insight into the relationship between metabolic profiles and genetic distances among plant accessions from which the hairy roots were derived, the third intron of the Tr2 gene was sequenced. Tr2 encodes tropinone reductase, which is involved in the synthesis of tropane alkaloids. The sequences were deposited in NCBI under Nos. MN017172 (accession Iraq), MN017173 (accession Iran), and MN017174 (accession Germany). The number of nucleotide differences per 1000 bp between the sequences from the accessions Iraq/Iran and Iraq/Germany were 21 and 23, respectively, while the accessions Iran and Germany differed in only 2 nucleotides. Thus the accessions Iran and Germany appeared more closely related than each of them to the accession Iraq. The similarity between sequences of a Tr2 gene of the accessions Iran and Germany was not reflected by the similarities of metabolic profiles of their hairy root exudates (Figure 10). Depending on cultivation conditions, the most similar profiles were Iraq/Germany and Iraq/Iran. The discrepancy between metabolic and genetic similarity is in line with the results of previous studies in other plant species [34,35,68]. In contrast, chemical profiles and microsatellite markers in *Citrus aurantium* were correlated [69]. *Hyoscyamus niger* is an attractive model to study the relationship between metabolic and genetic diversity in plants because it is a source of medicinally useful tropane alkaloids [70]. Attempts to produce tropane alkaloids in hairy root cultures of *Hyoscyamus niger* began decades ago [71] yet nothing is known about the genetic variation within the species. The availability of small-scale, inexpensive bioreactors will allow for comprehensive sampling of metabolic diversity in hairy root cultures of *H. niger*, which will facilitate selection of accessions for production purposes and enable studies of the relationship between metabolic and genetic diversity in this medical plant.

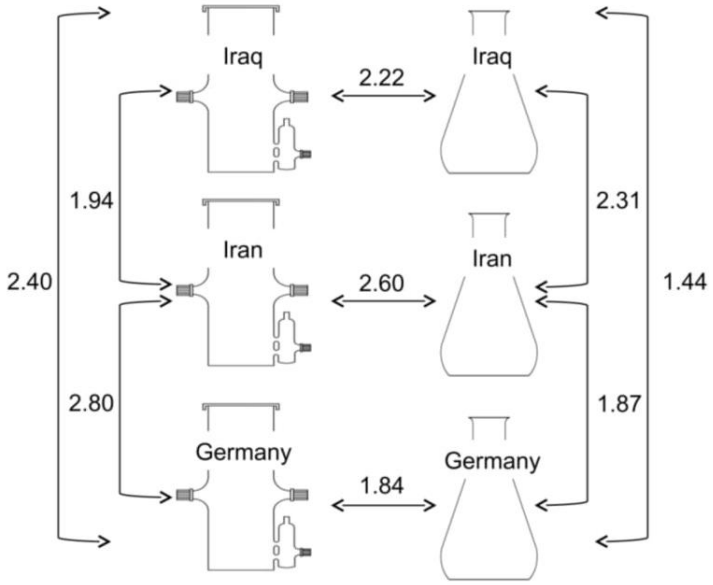

Figure 10. Euclidean distances between normalized metabolic profiles of hairy root exudates obtained from three accessions of *Hyoscyamus niger* (designated Iraq, Iran and Germany) grown in bioreactors and flask cultures.

4. Conclusions

The bioreactor designed in this work proved suitable for growing plants in sterile hydroponics, for cultivating hairy roots, and for collecting root and hairy root exudates. Hairy root cultures accumulated biomass at a comparable rate or faster in the bioreactor than in shaking flasks. The bioreactor outperformed shaking flasks regarding the diversity and quantity of hairy root exudates of *H. niger*.

Validation experiments lead to new findings regarding the metabolic diversity in hairy root exudates of *Hyoscyamus niger*, which turned out to be incongruent with the genetic relationships among

plant accessions. Salicylic acid secretion by hairy roots of *Hyoscyamus niger* varied widely among plant accessions and between cultivation conditions.

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6.2. Appendix B: Genetic Variation

6.2.1. Gel electrophoresis

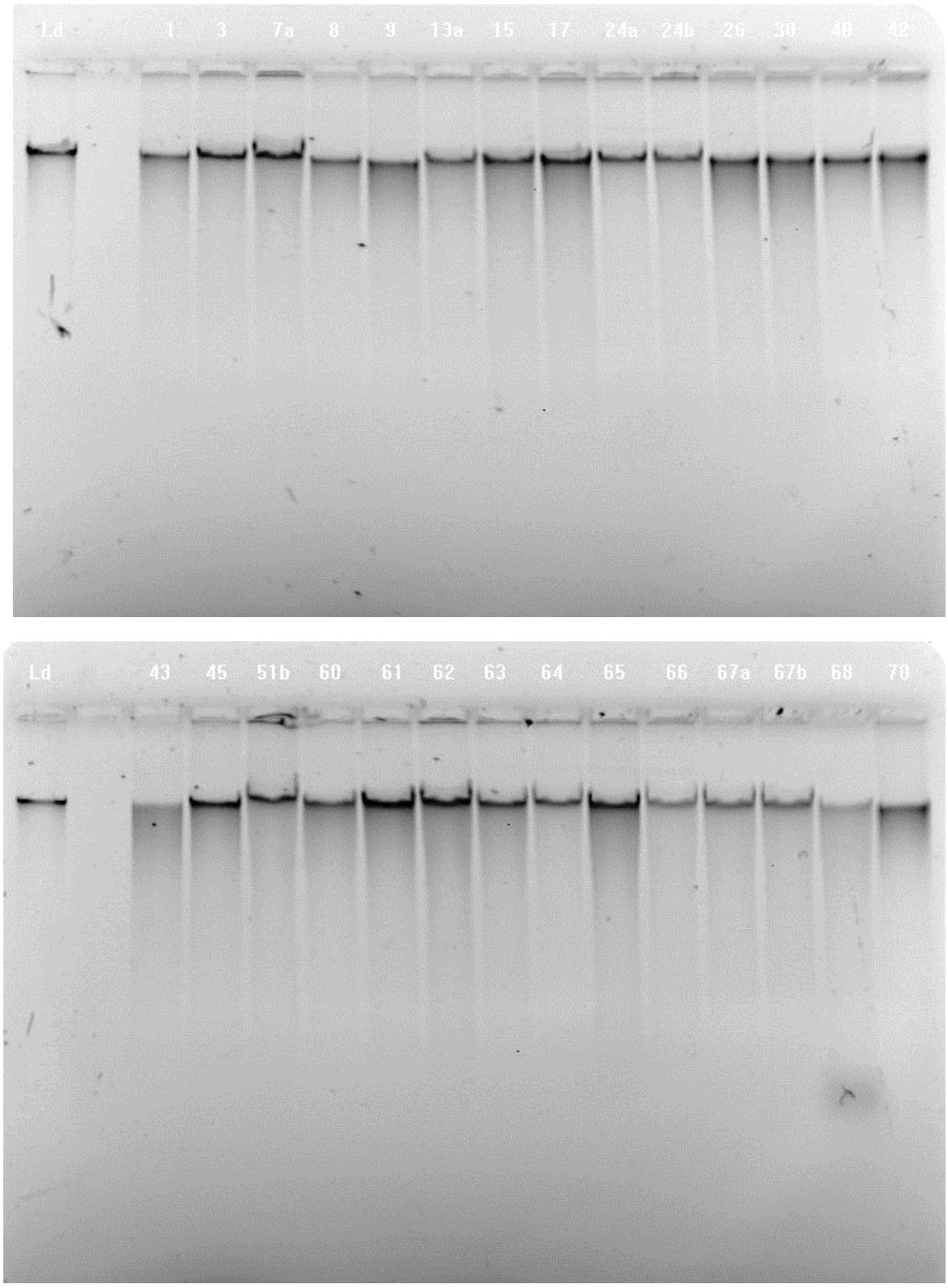

Figure 1: Gel electrophoresis of DNA extracts from seven samples of *Hyoscyamus niger* and twenty-five samples of *Sesamum indicum* samples.

6.2.2. PCR primer validation verification

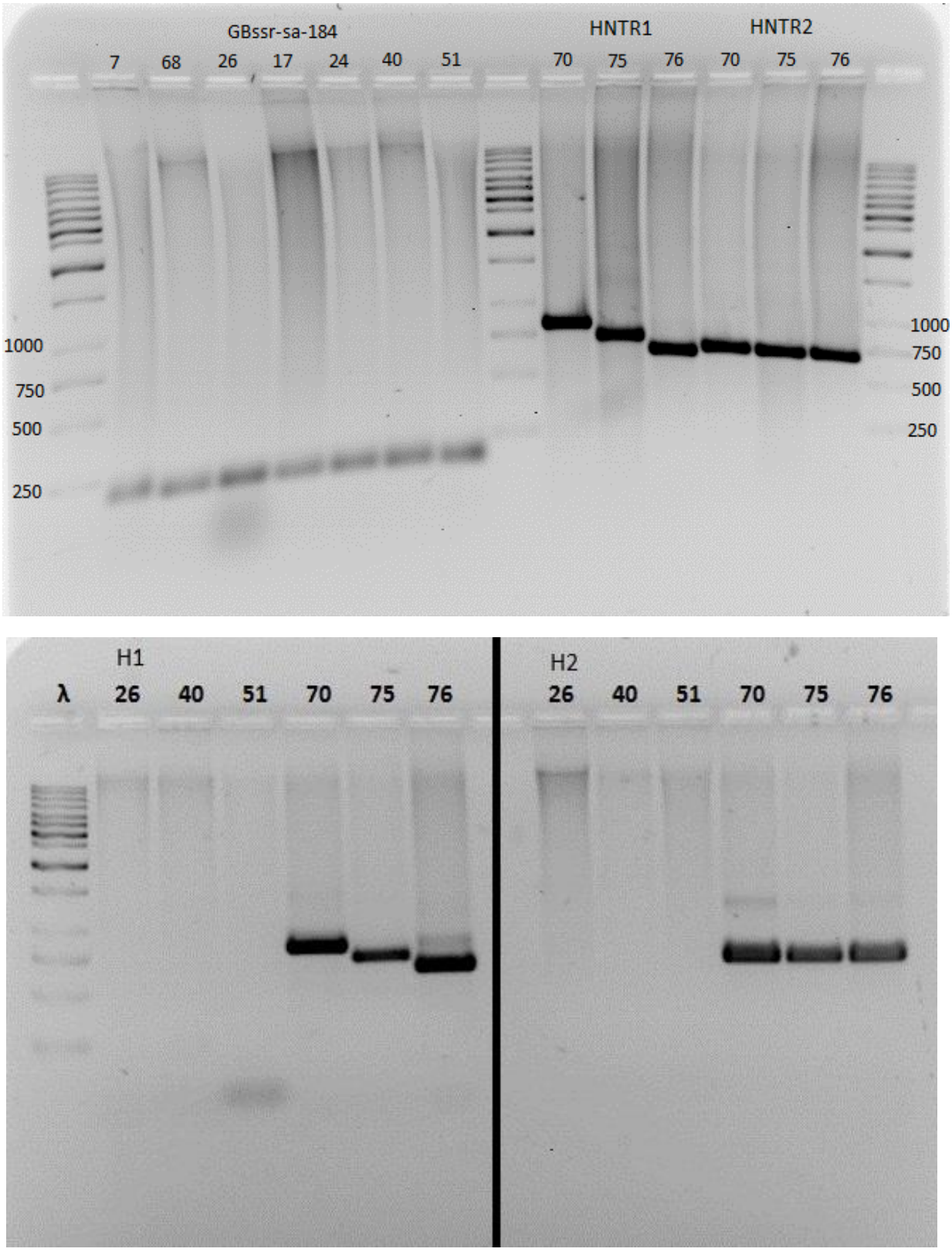

Figure 2: Product Gel electrophoresis of *Hyoscyamus niger* and *Sesamum indicum* HNTR1 and HNTR 2 and *GBssr-sa*-184 genes.

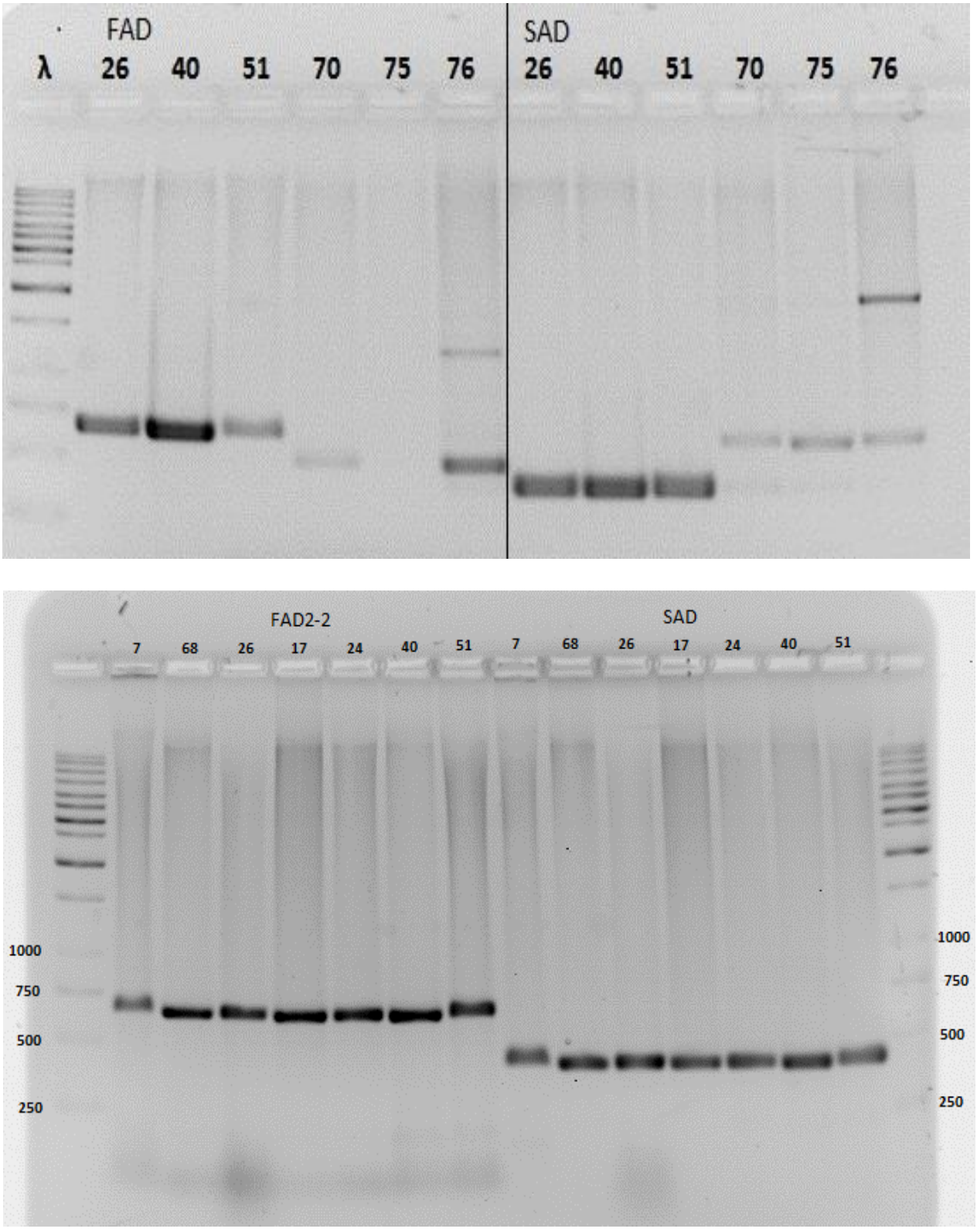

Figure 3: PCR product gel electrophoresis of *Sesamum indicum FAD* and *SAD* genes.

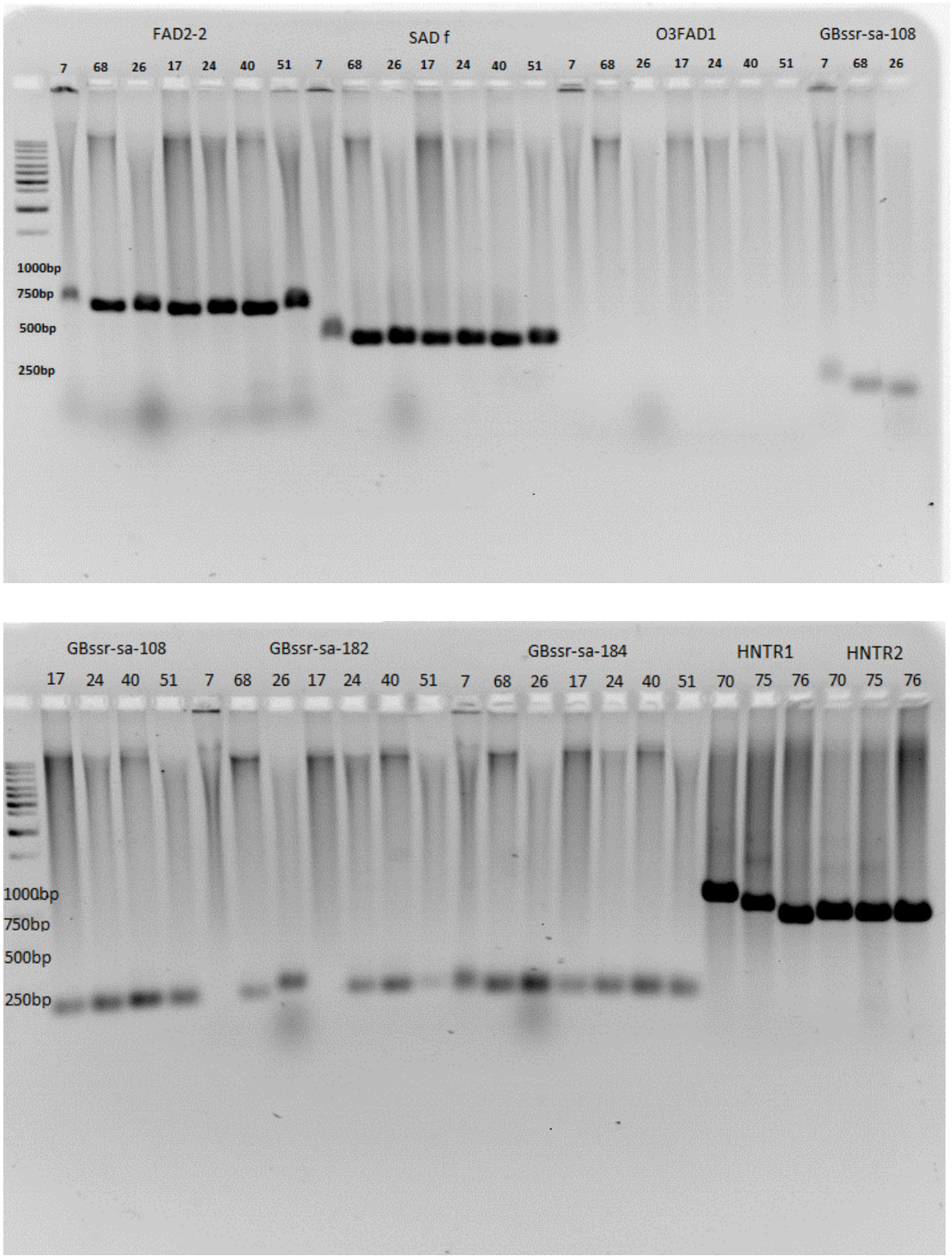

Figure 4: PCR product gel electrophoresis of *Sesamum indicum FAD, SAD, O3FAD1, GBssr-sa-108, GBssr-sa-182, GBssr-sa-184* genes, and *Hyoscyamus niger HNTR1, HNTR2* genes

6.2.3. DNA Sequencing results

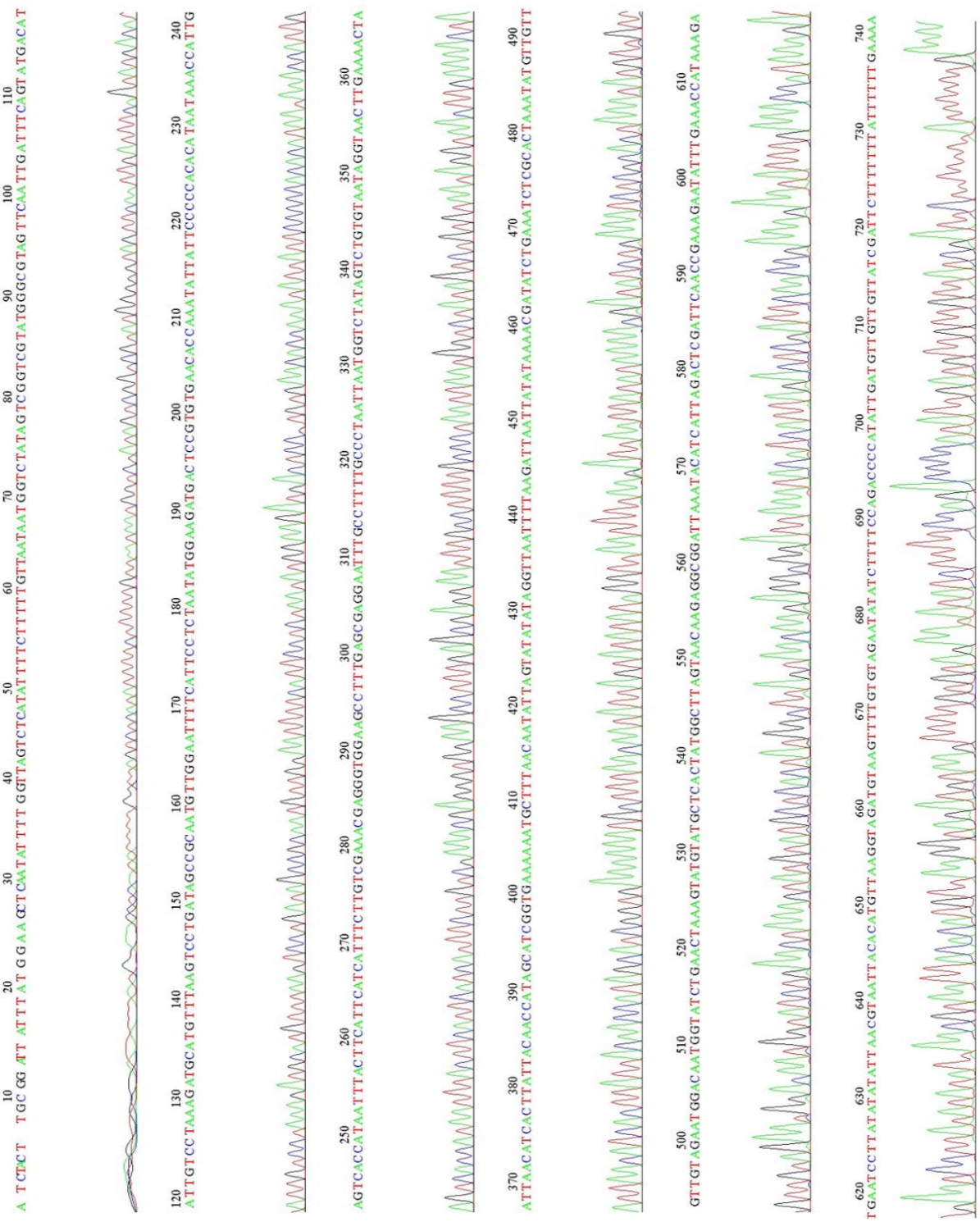

Figure 5: Sequencing result of KRI Hawraman accession, *HNTR1* gene.

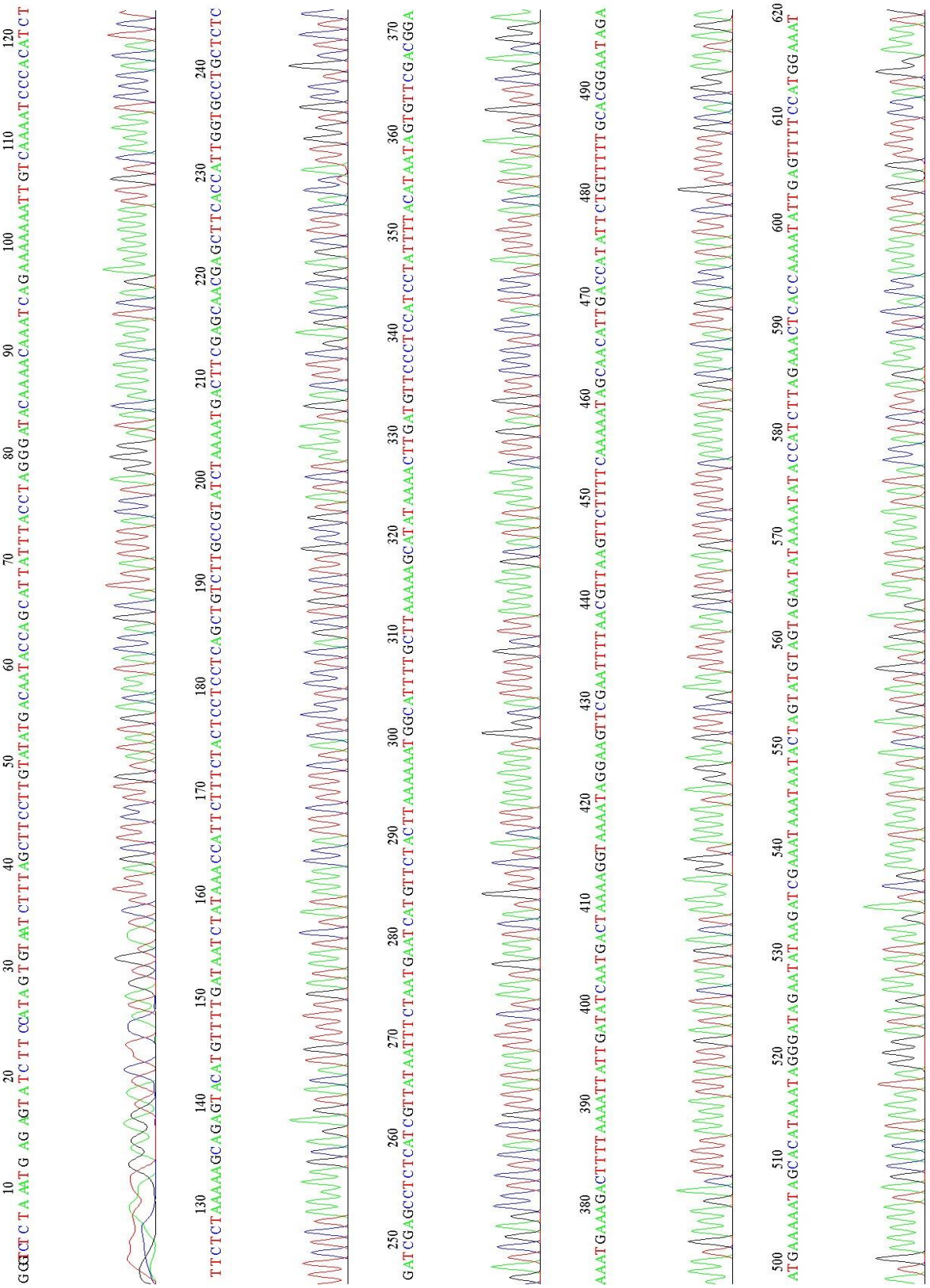

Figure 6: Sequencing result of KRI Hasanawa accession, *HNTR2* gene.

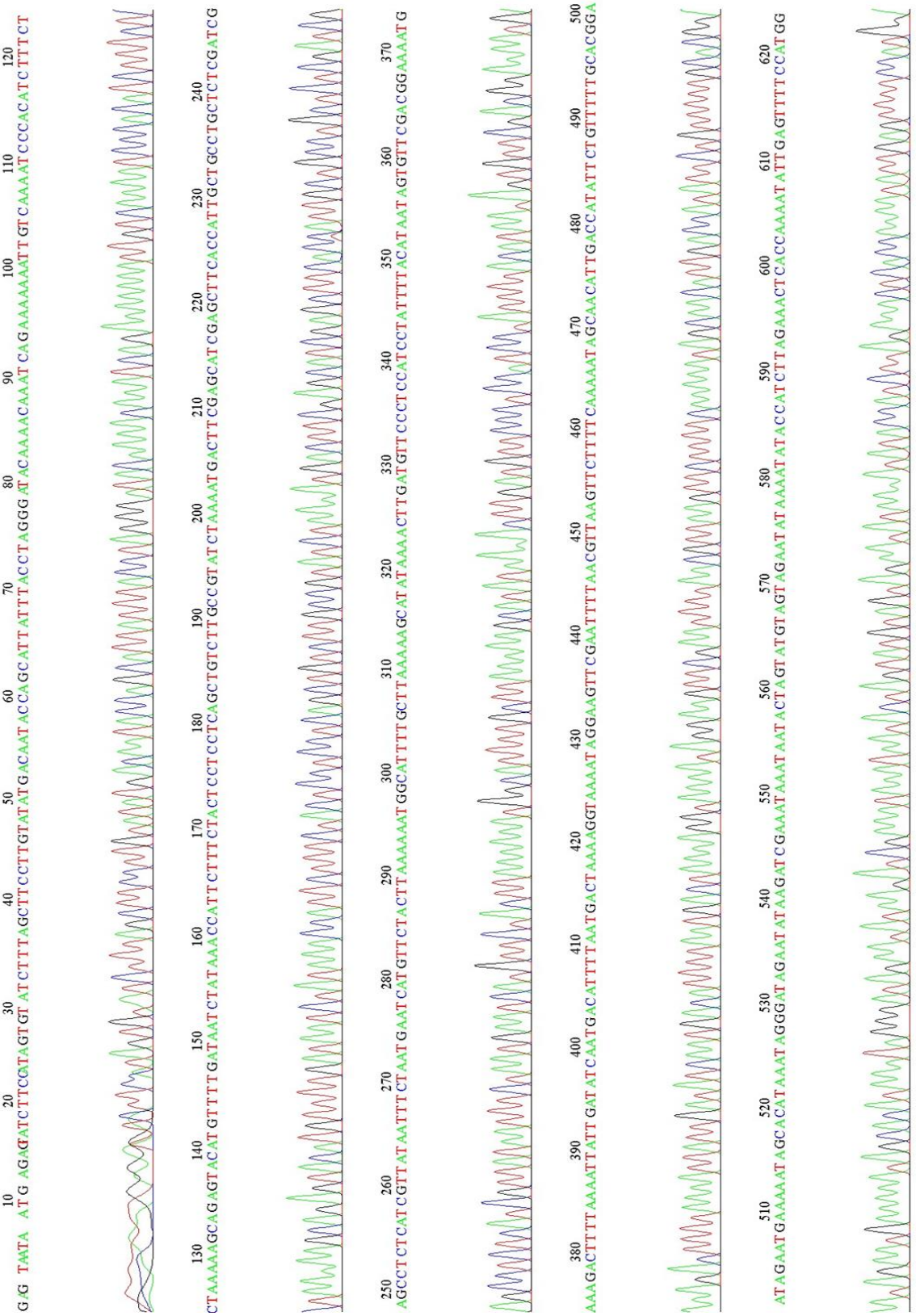

Figure 7: Sequencing result of KRI Pinjwen accession, *HNTR2* gene.

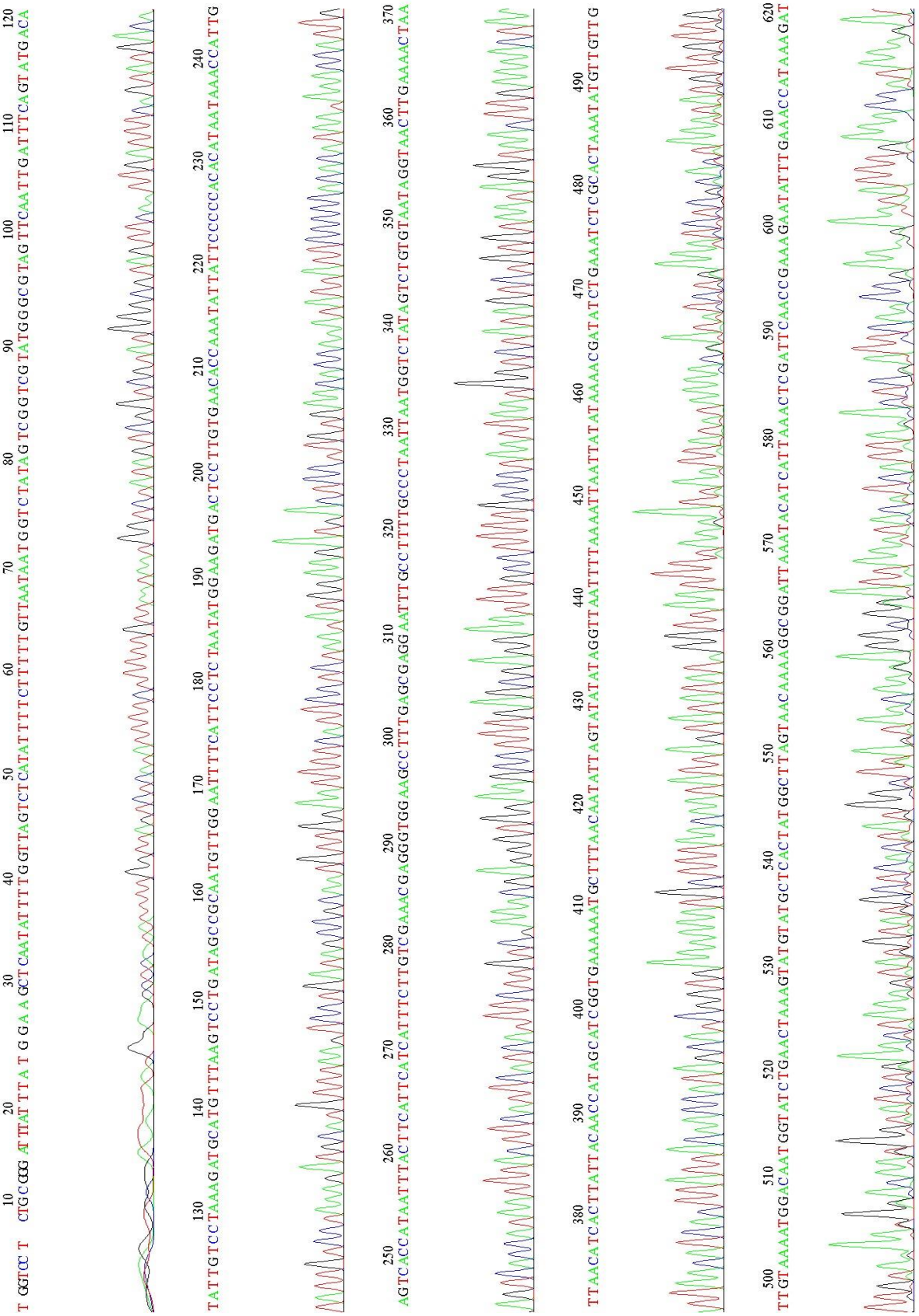

Figure 8: Sequencing result of KRI Darae Mar accession, *HNTR2* gene.

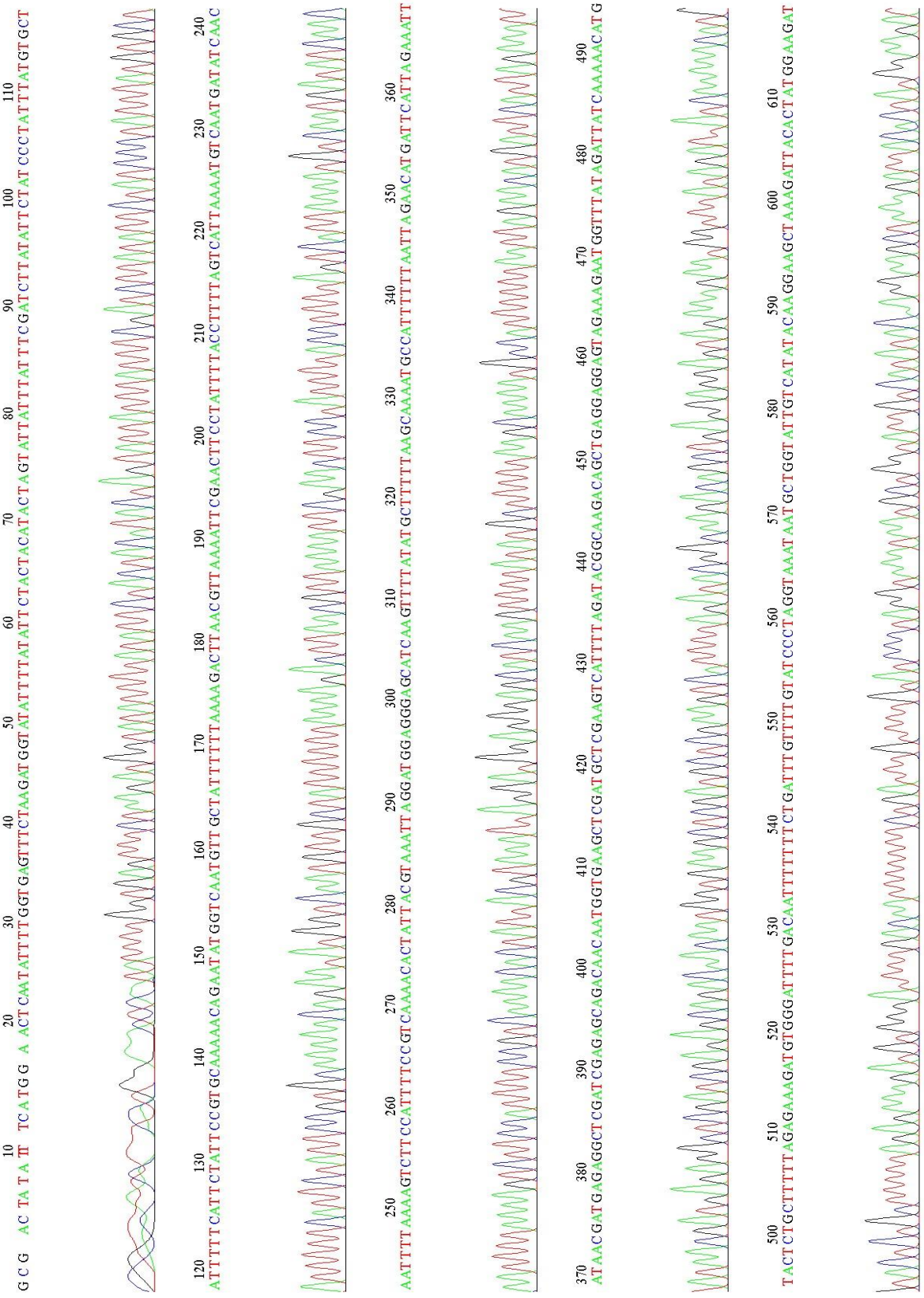

Figure 9: Sequencing result of Iran Takht accession, *HNTR2* gene.

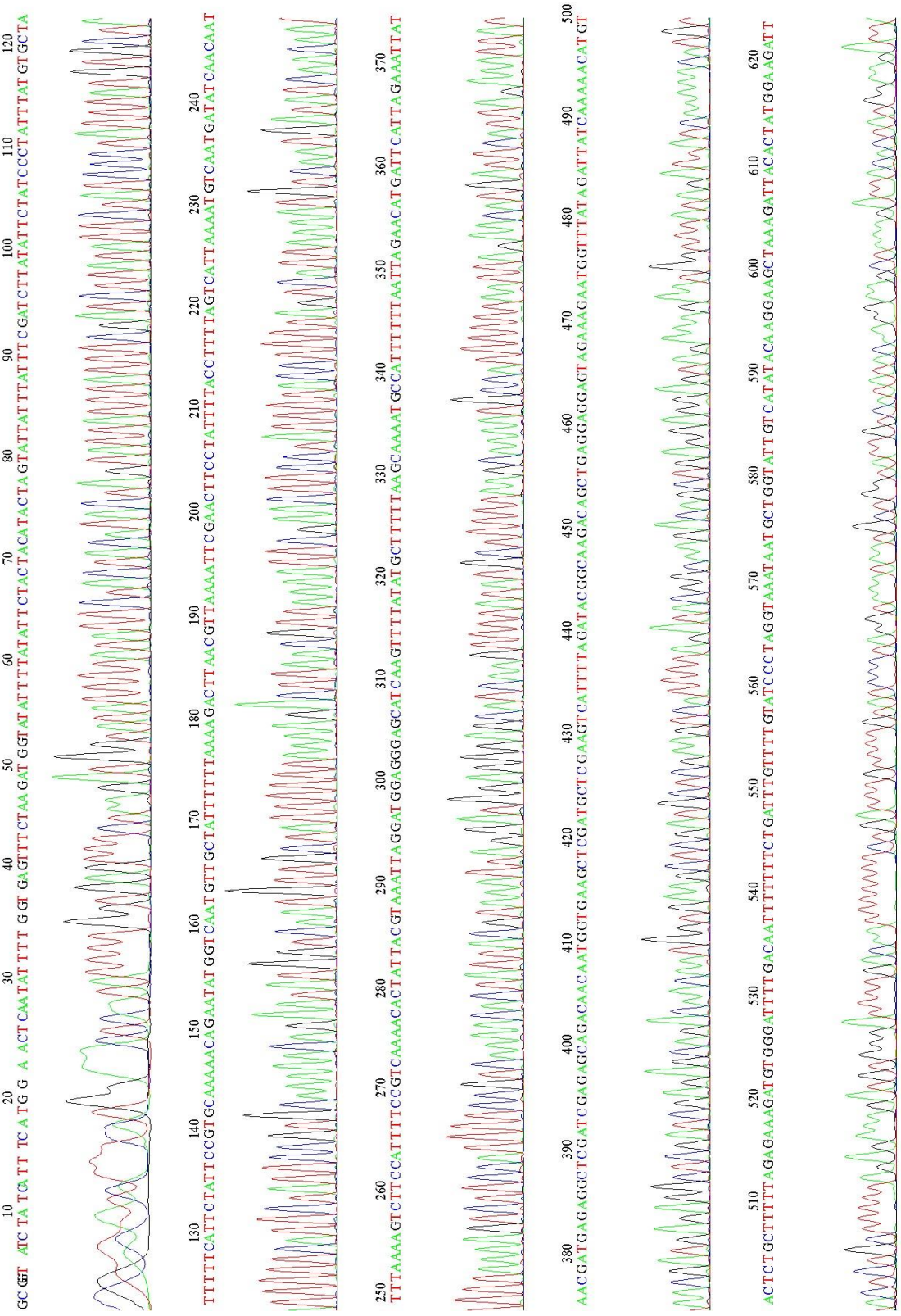

Figure 10: Sequencing result of Iran Isfahan accession, *HNTR2* gene.

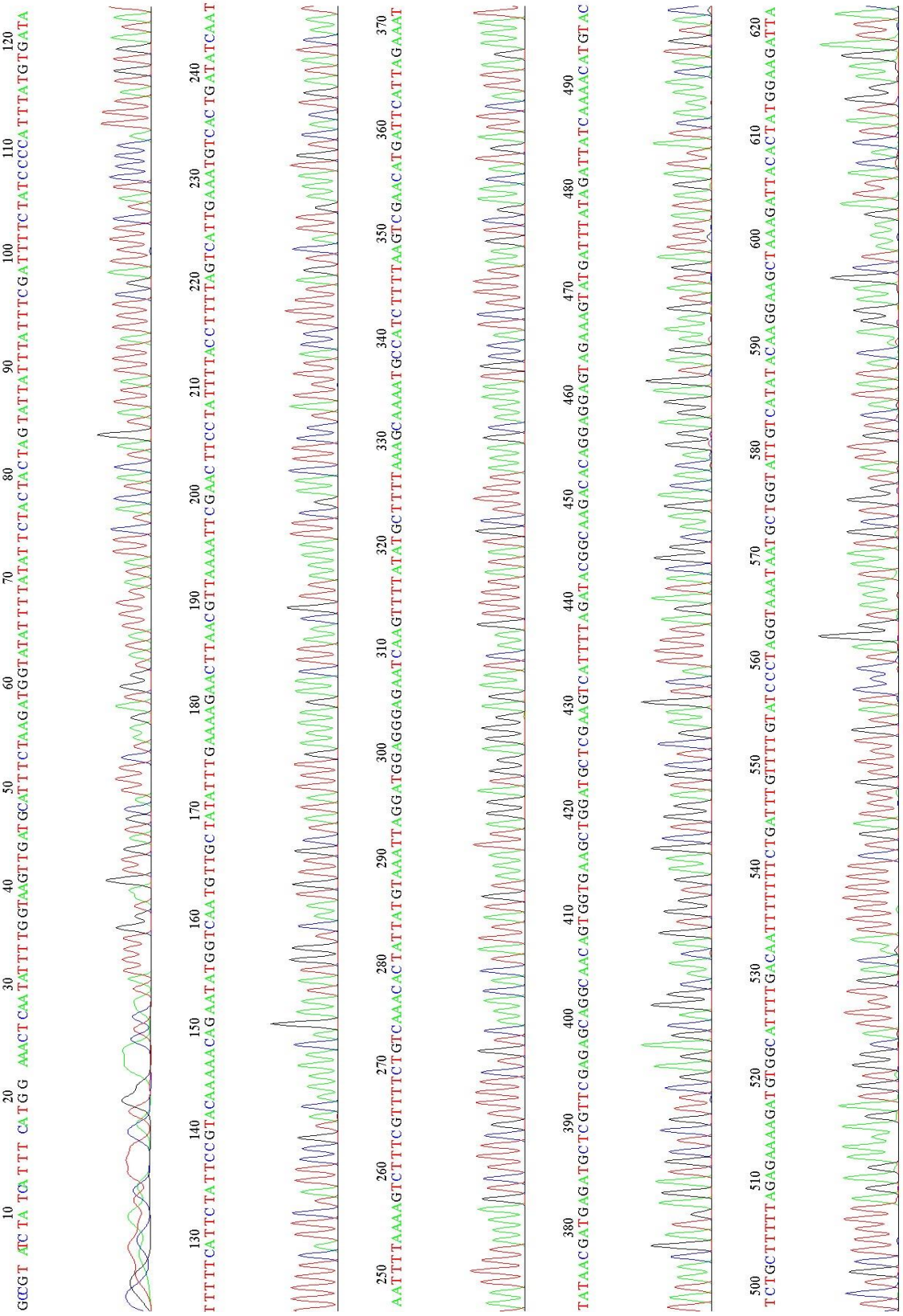

Figure 11: Sequencing result of Germany 1 accession, *HNTR2* gene.

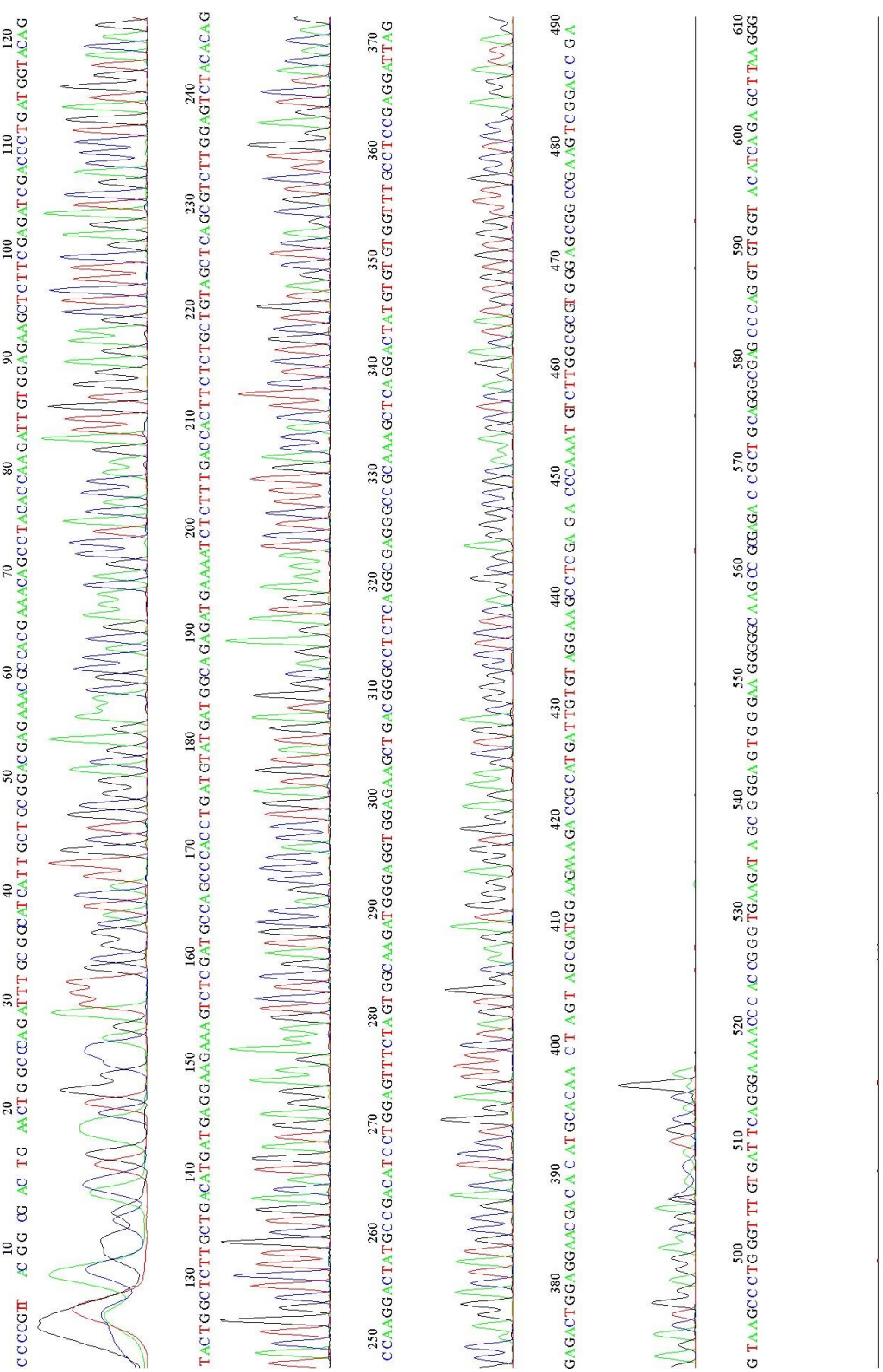

Figure 12: Sequencing result of India accession, *SAD* gene.

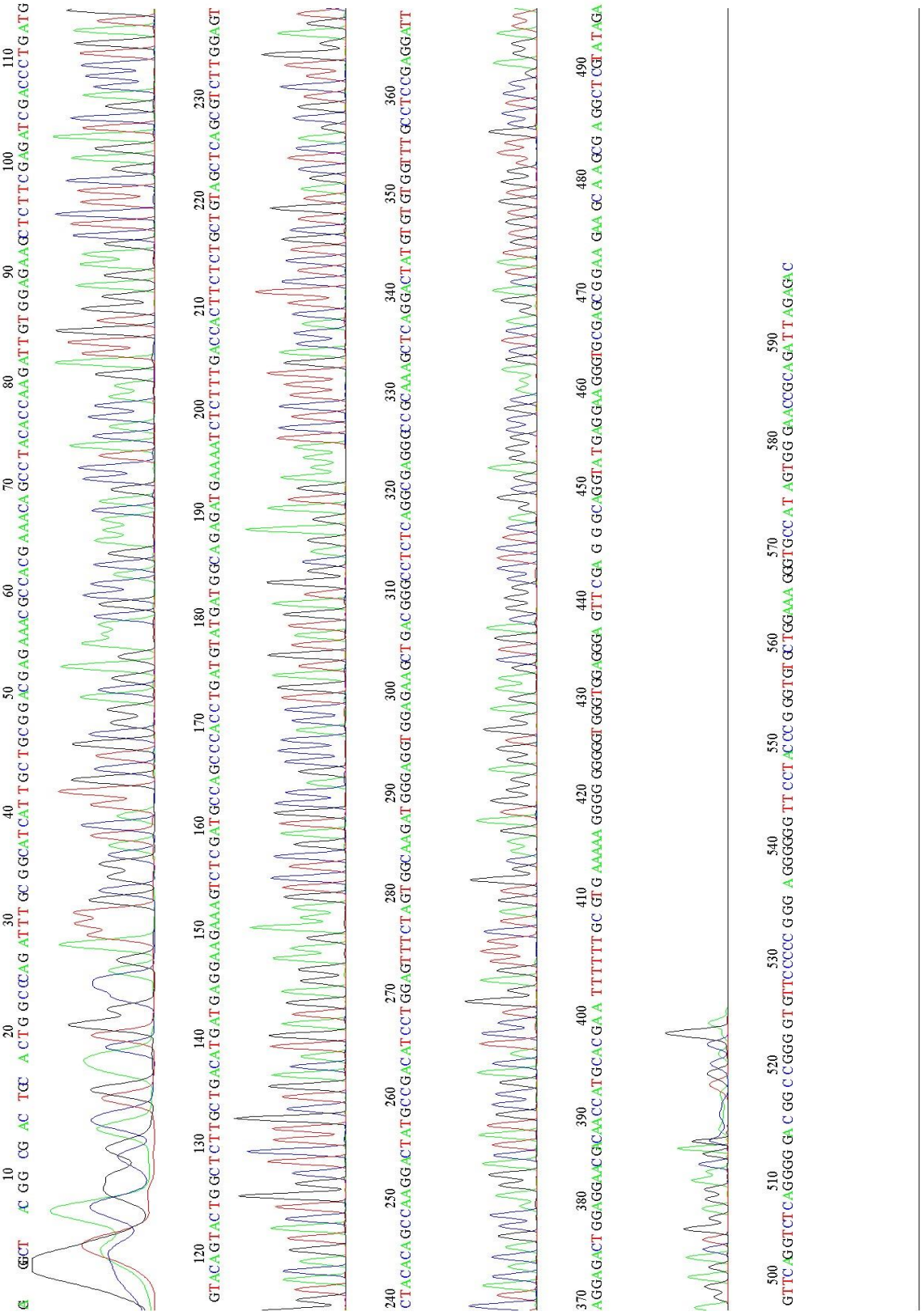

Figure 13: Sequencing result of India 3 accession, *SAD* gene.

Figure 14: Sequencing result of Japan 2 accession, *SAD* gene.

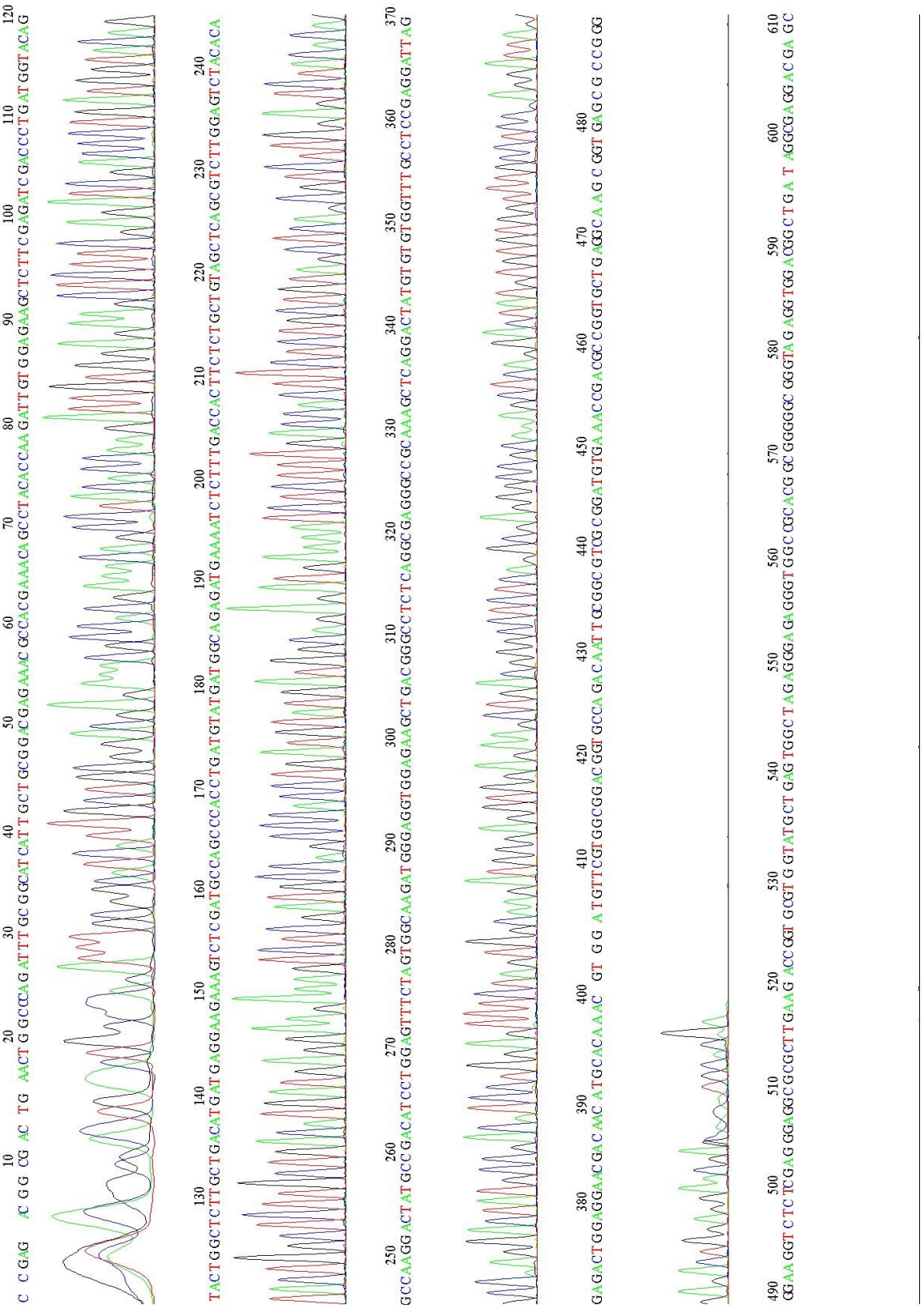

Figure 15: Sequencing result of Maporal accession, *SAD* gene.

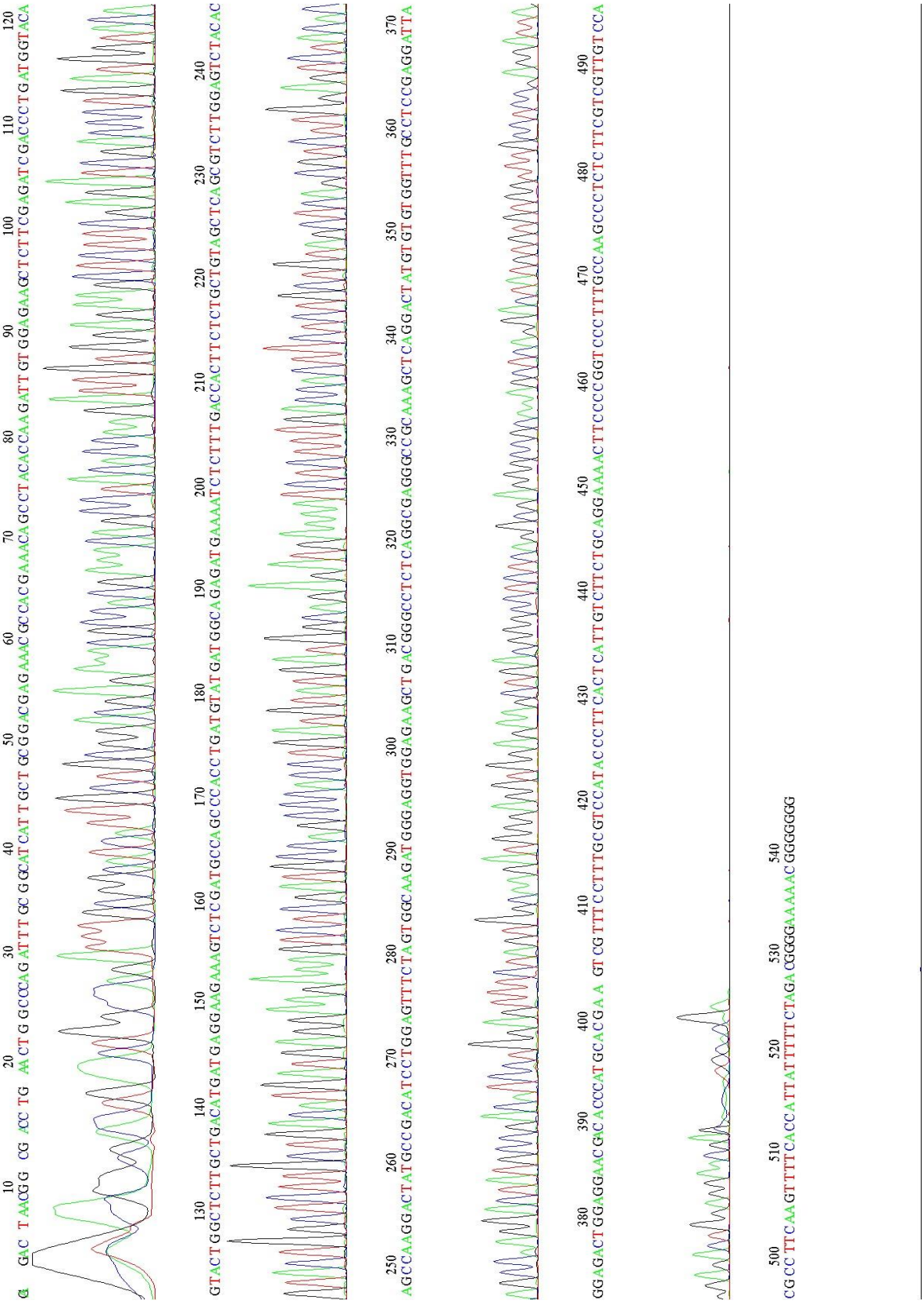

Figure 16: Sequencing result of Inamar accession, *SAD* gene.

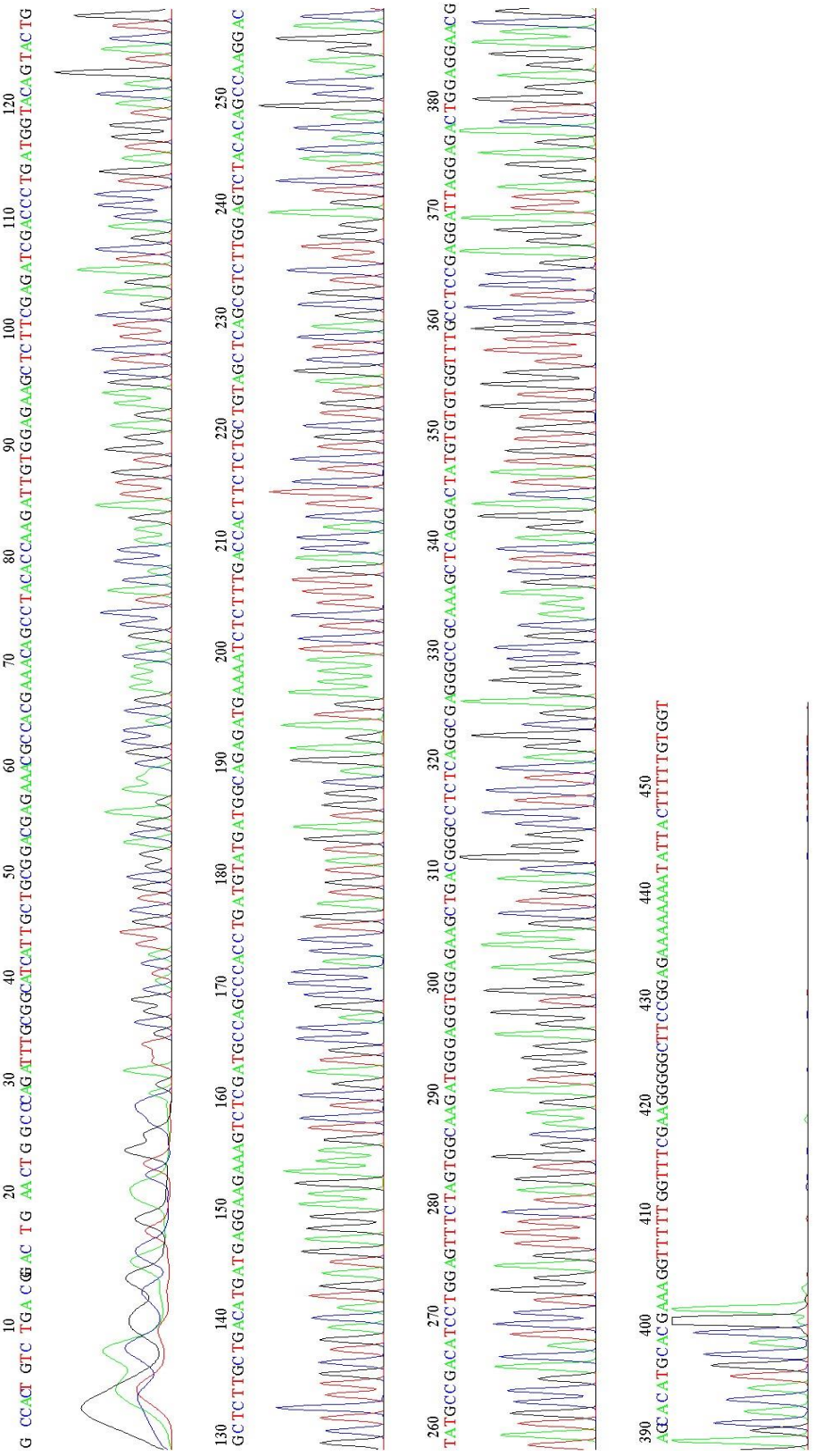

Figure 17: Sequencing result of USLA 1 accession, *SAD* gene.

D

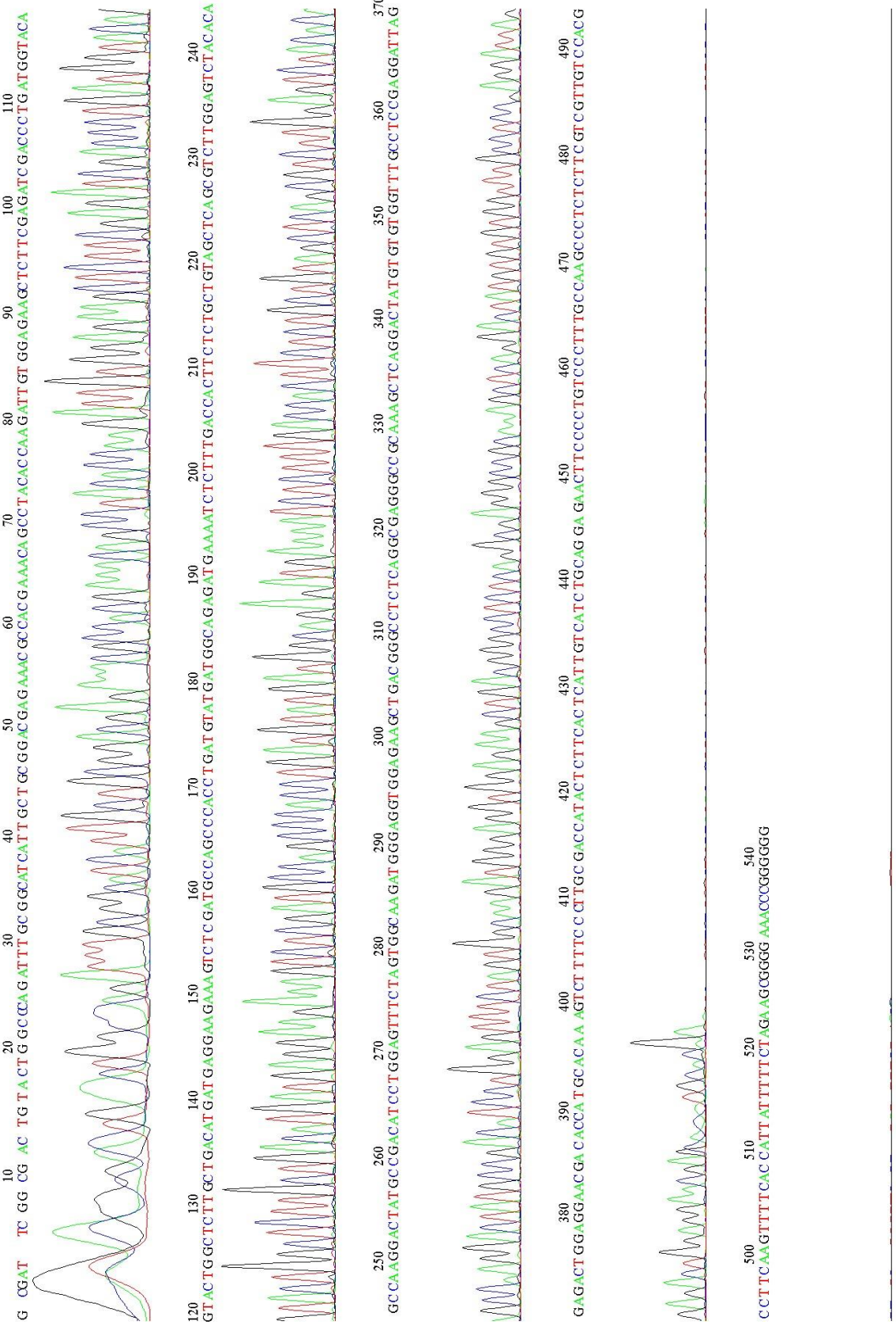

Figure 18: Sequencing result of Sumer S1a accession, *SAD* gene.

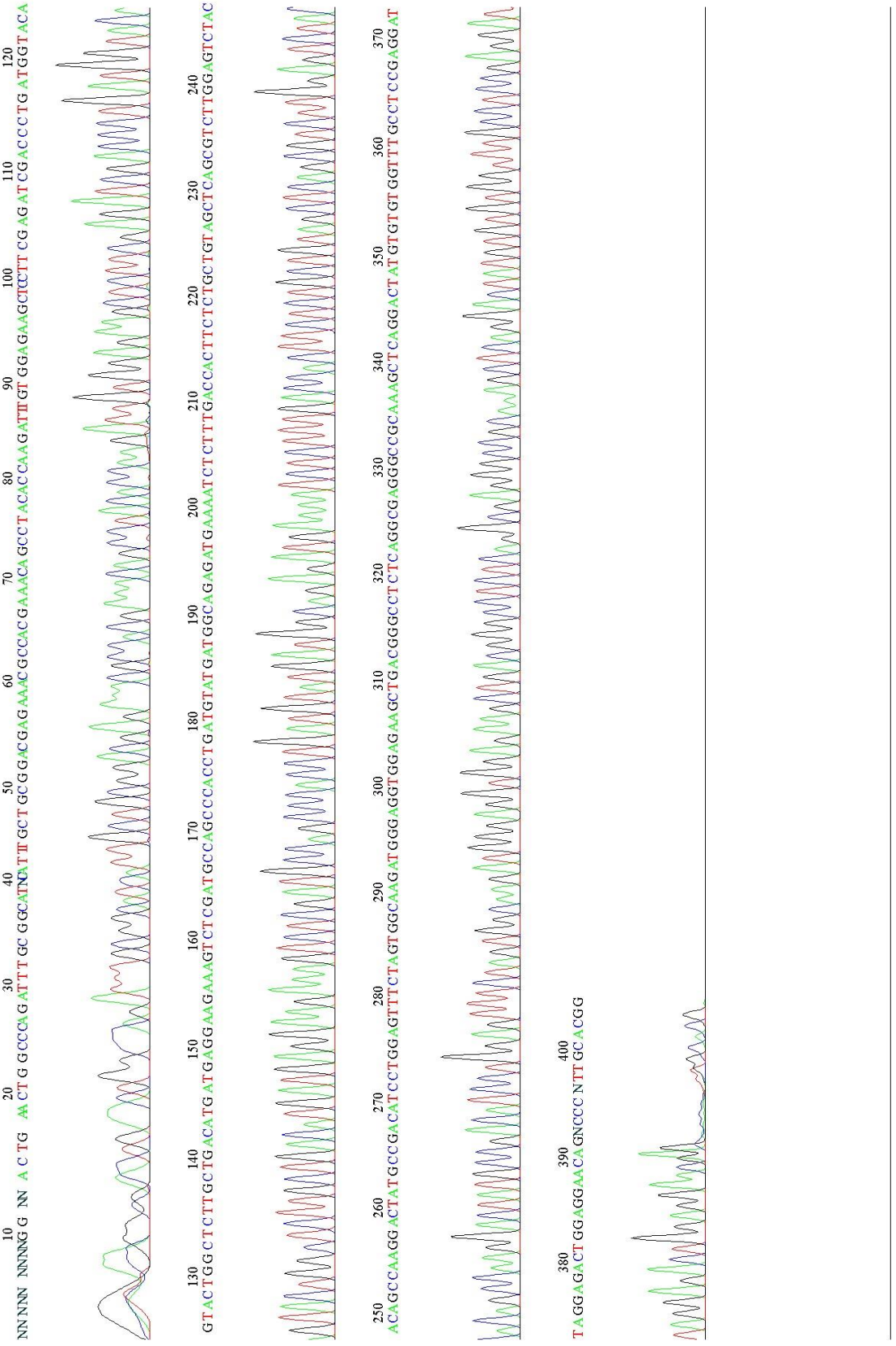

Figure 19: Sequencing result of Sumer S2a accession, *SAD* gene.

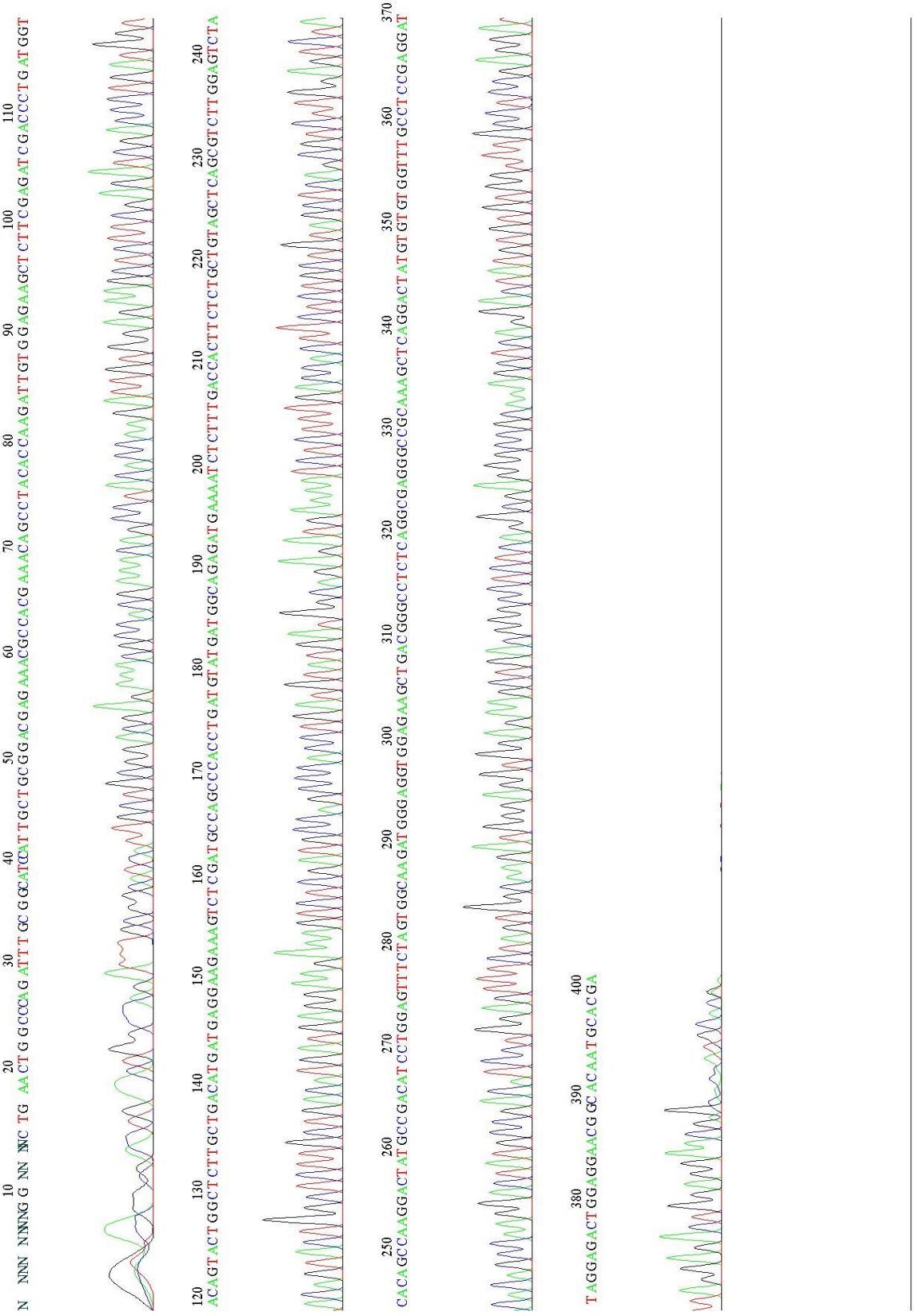

Figure 20: Sequencing result of KRI local Ranya 1 accession, *SAD* gene.

6.3. Appendix C: Chemical Profiling

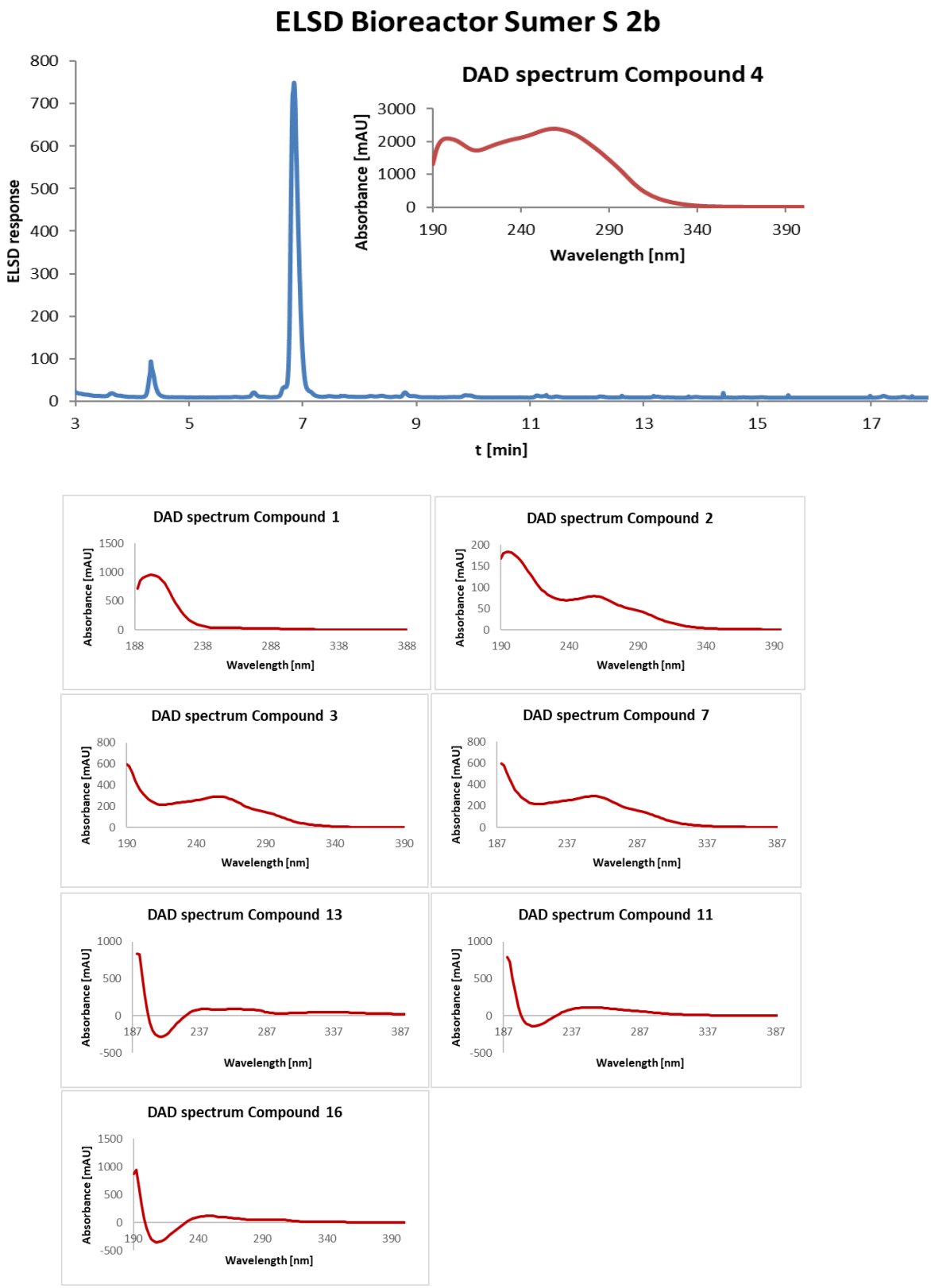

Figure 21: ELSD, DAD analysis of *Sesamum indicum* Sumer S2b accession Bioreactor exudate

Table 1: Lignan content in Sesame accessions Hairy Root

Plant accessions	Sesamol [mg/kg]	Pinoresinol [mg/kg]	Sesaminol [mg/kg]	Sesamin [mg/kg]	Sesamolin [mg/kg]
India 1	0.037	0.021	0.035	1.501	0.054
India 3	0.037	0.021	0.035	17.949	0.054
India 7	0.037	0.021	0.035	9.075	0.054
India 8	0.037	0.021	0.035	3.859	0.054
Korea 1	0.037	0.021	0.035	5.658	0.054
Turkey	0.037	0.021	0.035	1.668	0.054
Syria	0.037	0.021	0.035	7.776	0.054
Sudan 2	0.037	0.021	0.035	10.310	0.054
Africa 3	0.037	0.021	0.035	4.050	0.054
Japan 2	0.037	0.021	0.035	2.694	0.054
China 2	0.037	0.021	0.035	12.403	0.054
Vzla 51	0.037	0.021	0.035	5.641	0.054
UCV-1	0.037	0.021	0.035	6.309	0.054
Maporal	0.037	0.021	0.035	2.251	0.054
Inamar	0.037	0.021	0.035	11.277	0.054
UCLA 1	0.037	0.021	0.035	0.029	0.054
KRG introduced S1a	0.037	0.021	0.035	0.148	0.054
KRG introduced S1b	0.037	0.021	0.035	0.119	0.054
KRG introduced S2a	0.037	0.021	0.035	0.029	0.054
KRG introduced S2b	0.037	0.021	0.035	0.029	0.054
KRG local Hawler 1	0.037	0.021	0.035	0.029	0.054
KRG local Ranya 1	0.037	0.021	0.035	2.945	0.054
KRG local Hawler 2	0.037	0.021	0.035	0.029	0.054
KRG local Ranya 2	0.037	0.021	0.035	0.029	0.054
Iran 1	0.037	0.021	0.035	10.734	0.054

Table 2: Lignan content in Sesame accessions Roots

Plant accessions	Pinoresinol [mg/kg]	Sesaminol [mg/kg]	Sesamin [mg/kg]	Sesamolin [mg/kg]
India 1	0.110	0.035	0.176	0.054
India 3	0.074	0.035	1.276	0.054
India 7	0.021	0.035	0.578	0.054
India 8	0.027	0.035	2.632	0.054
Korea 1	0.040	0.035	0.418	0.054
Turkey	0.049	0.035	1.035	0.054
Syria	0.069	0.035	1.067	0.054
Sudan 2	0.040	0.035	0.406	0.054
Africa 3	0.080	0.035	1.509	0.054
Japan 2	0.034	0.035	0.429	0.054
China 2	0.593	0.035	0.130	0.054
Vzla 51	0.075	0.035	0.351	0.054
UCV-1	0.089	0.035	0.097	0.054
Maporal	0.034	0.035	0.050	0.054
Inamar	0.119	0.035	0.187	0.054
UCLA 1	0.673	0.035	0.040	0.054
KRG introduced S1a	0.089	0.035	0.080	0.054
KRG introduced S1b	0.137	0.035	0.060	0.054
KRG introduced S2a	0.065	0.035	2.974	0.054
KRG introduced S2b	0.021	0.035	0.151	0.054
KRG local Hawler 1	0.062	0.035	0.437	0.054
KRG local Ranya 1	0.047	0.035	1.566	0.054
KRG local Hawler 2	0.021	0.035	0.181	0.054
KRG local Ranya 2	0.048	0.035	1.529	0.054
Iran 1	0.044	0.035	8.697	0.054

Table 3: Lignan content in Sesame accessions Seeds

Plant accessions	Sesamin	Sesamolin	Sesaminol	Pinoresinol
India 1	0.75	0.86	0.3	0.29
India 3	1.7	1.39	0.64	1.69
India 7	1.48	0.93	0.44	0.36
India 8	1.68	1.59	0.85	1.61
Korea 1	1.73	1.26	0.24	1.09
Turkey	1.85	1.74	0.21	0.11
Syria	1.44	0.37	0.19	0.08
Sudan 2	1.19	0.76	0.12	0.13
Africa 3	0.94	1.01	0.09	0.05
Japan 2	1.75	1.36	0.15	0.13
China 2	1.39	1.38	0.34	0.19
Vzla 51	1.19	1.33	0.23	0.32
UCV-1	1.5	1.33	0.17	0.44
Maporal	1.28	0.75	0.44	0.32
Inamar	1.57	1.08	0.43	0.62
UCLA-1	1.61	0.96	0.18	0.27
Sumer S1a	0.77	0.7	0.07	0.06
Sumer S1b	0.49	0.71	0.08	0.12
Sumer S2a	1.47	0.8	0.11	0.12
Sumer S2b	1.98	0.91	0.13	0.15
KRI local Hawler 1	2.1	1.06	0.24	0.17
KRI local Ranya 1	2.11	0.9	0.2	0.1
KRI local Hawler 2	2.09	0.94	0.22	0.12
KRI local Ranya 2	1.92	0.84	0.11	0.08
Iran 1	1.59	1.01	0.08	0.08

6.4. Appendix C: Biomedical applications & *Caenorhabditis elegans*

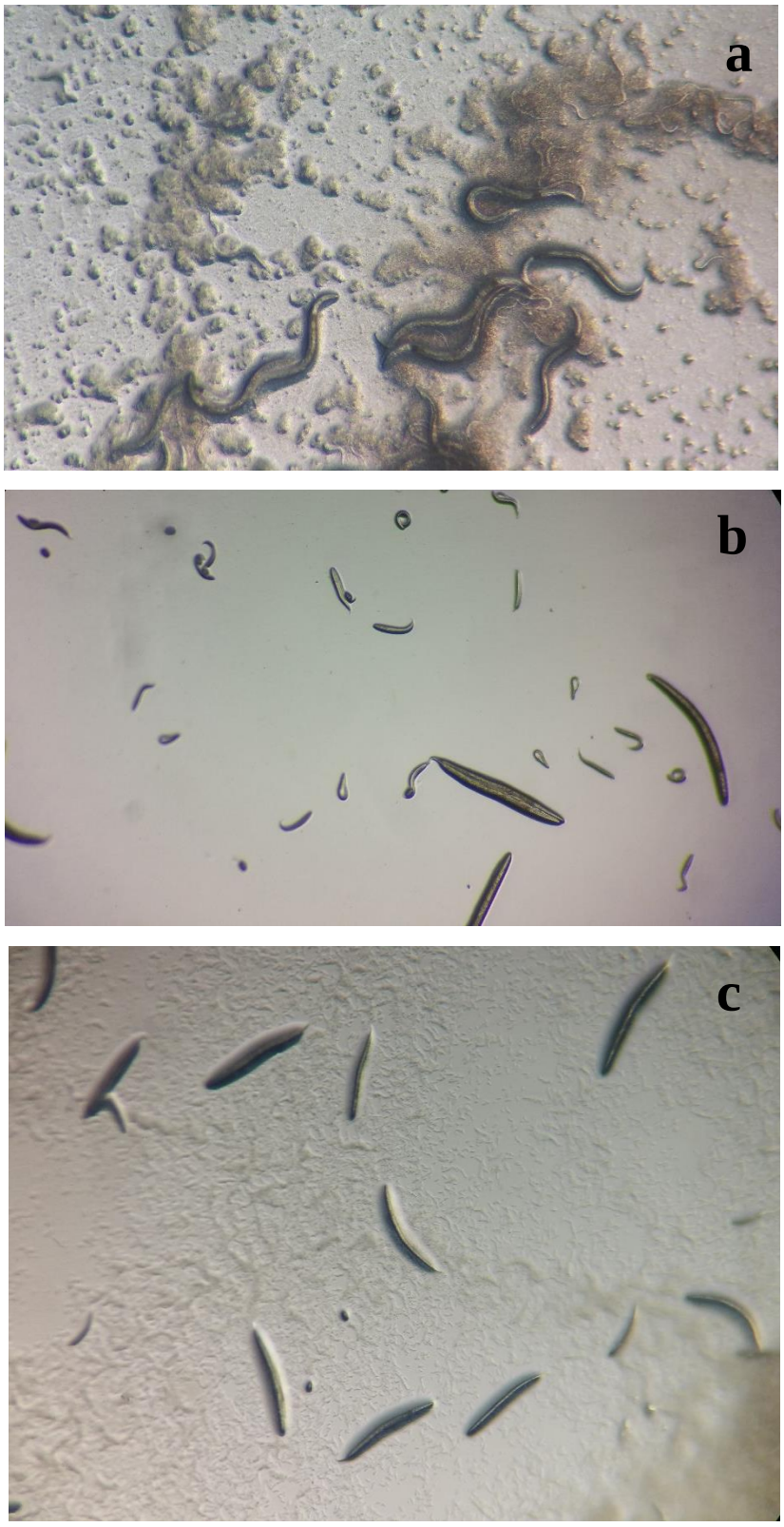

Figure 22: Thermal release mortality result; (b, c) compared with control (a)

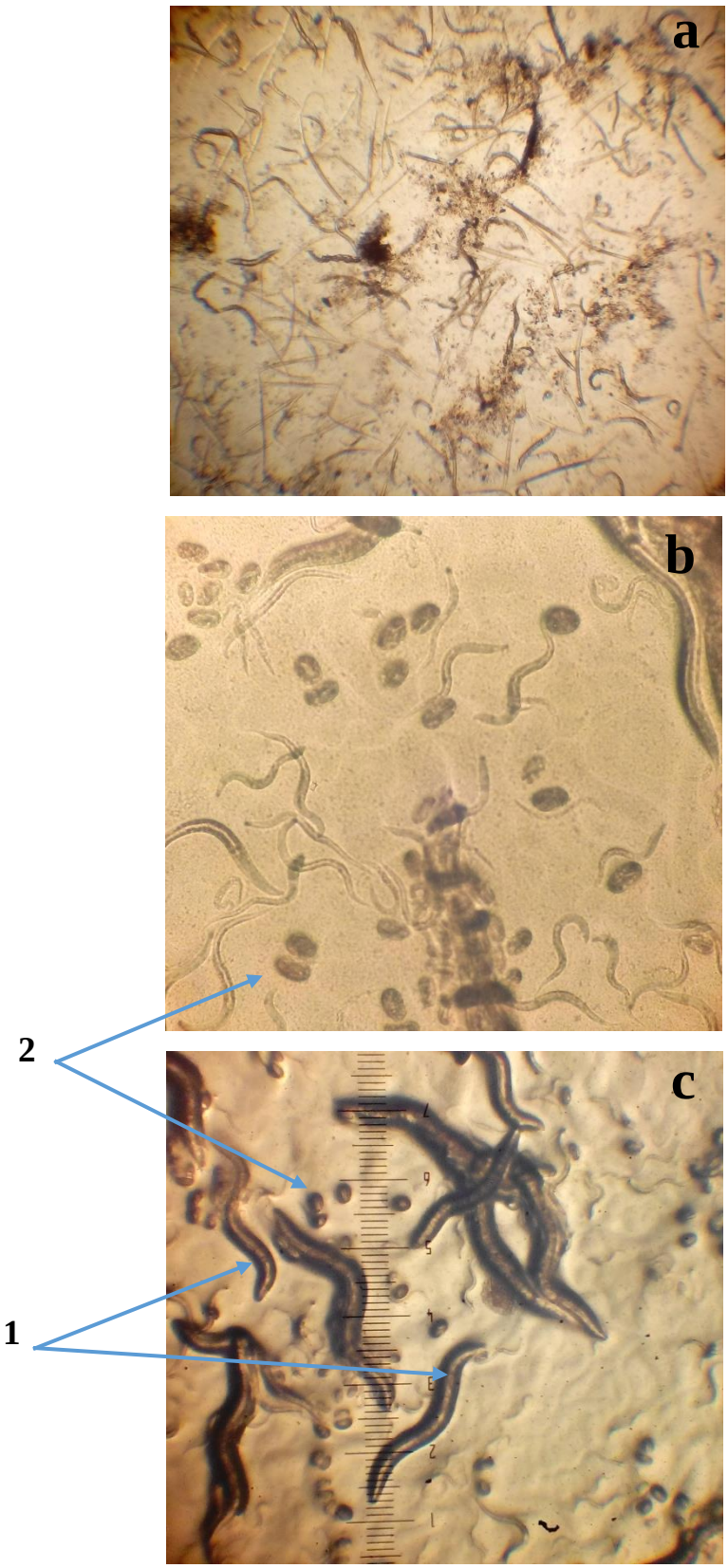

Figure 23: ***Caenorhabditis elegans*****; a.** ***Hyoscyamus niger*** **methanol extract mortality result. b, c (1) Adult** ***C. elegans*** **with (2) mature egg.**

6.5. Seed germination and plant flowering

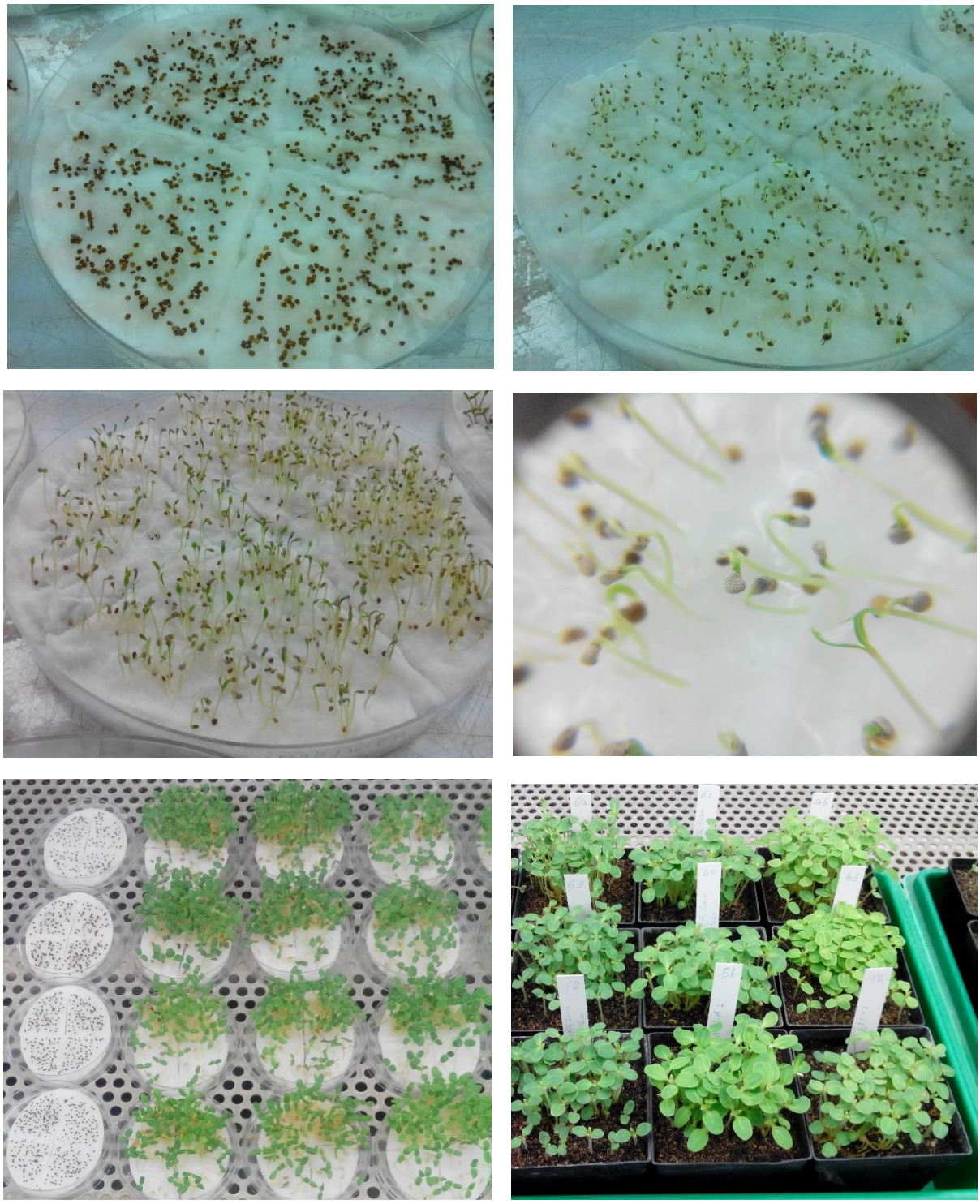

Figure 24: ***Hyoscyamus niger*** **and** ***Sesamum indicum*** **Seed germination**

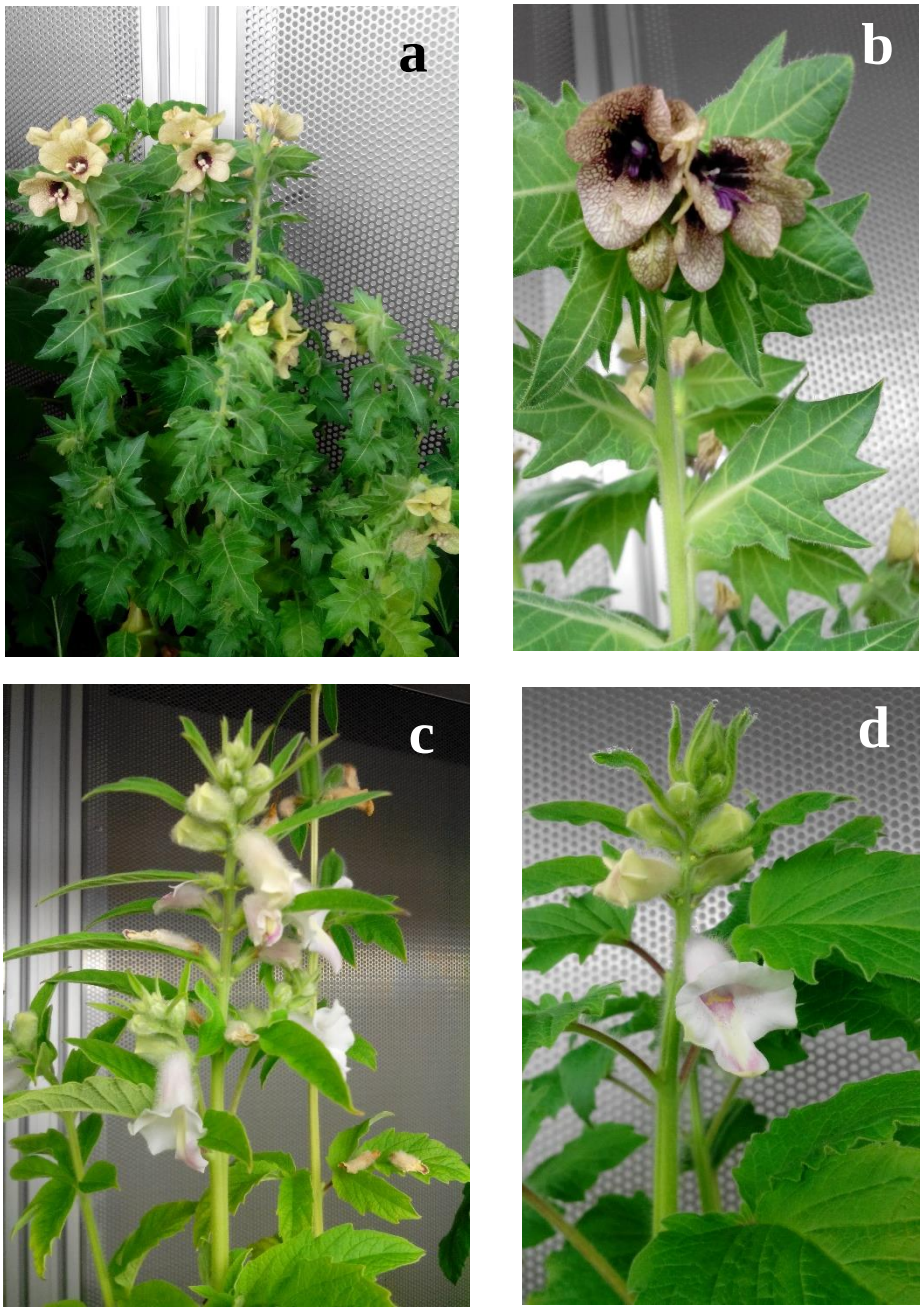

Figure 25: *Hyoscyamus niger* (a, b) and *Sesamum indicum* (c, d), flowering stage

6.6. Hairy Root Culture

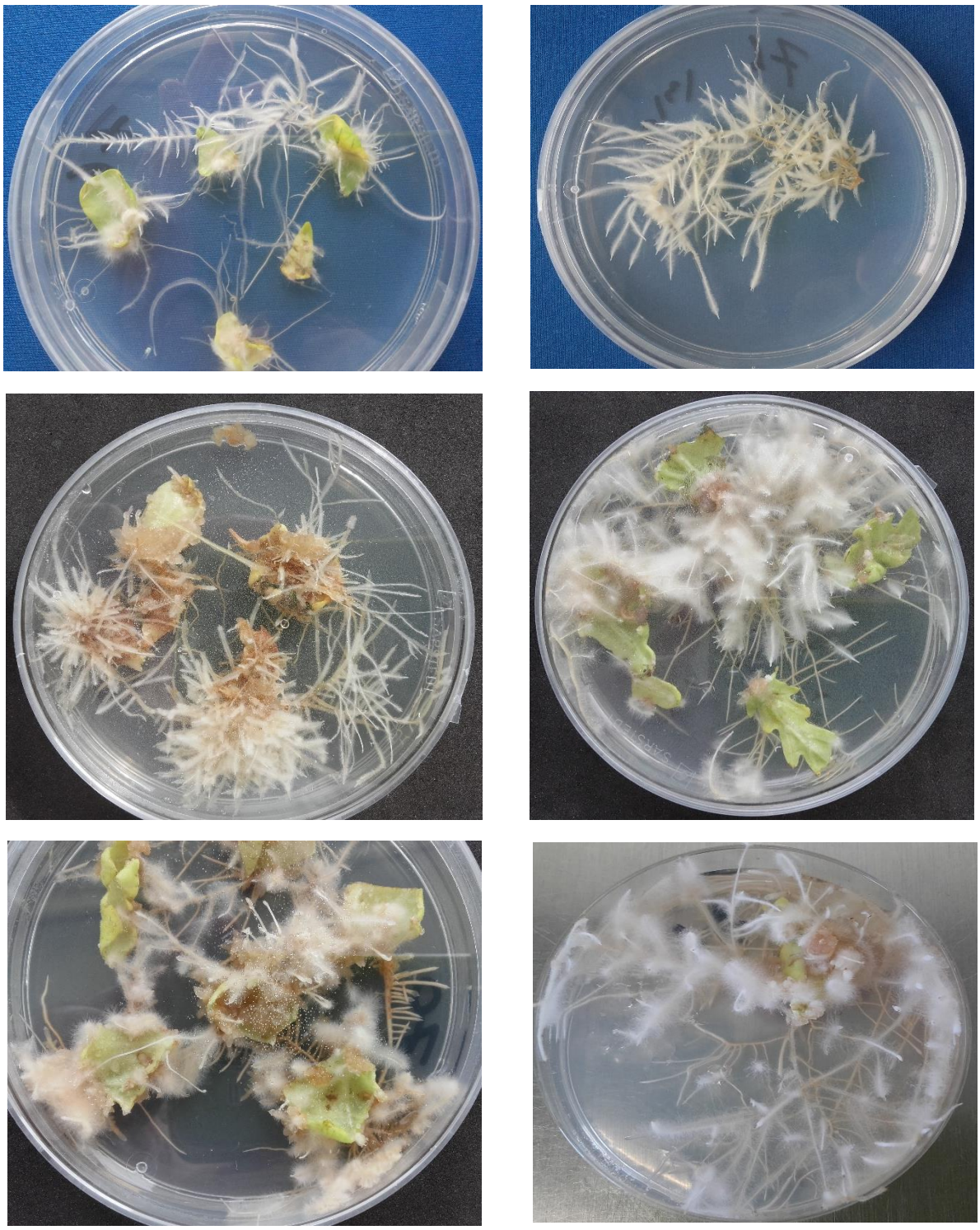

Figure 26: *Hyoscyamus niger* and *Sesamum indicum* hairy root induction

Figure 27: *Hyoscyamus niger* and *Sesamum indicum* hairy root with different morphological structure

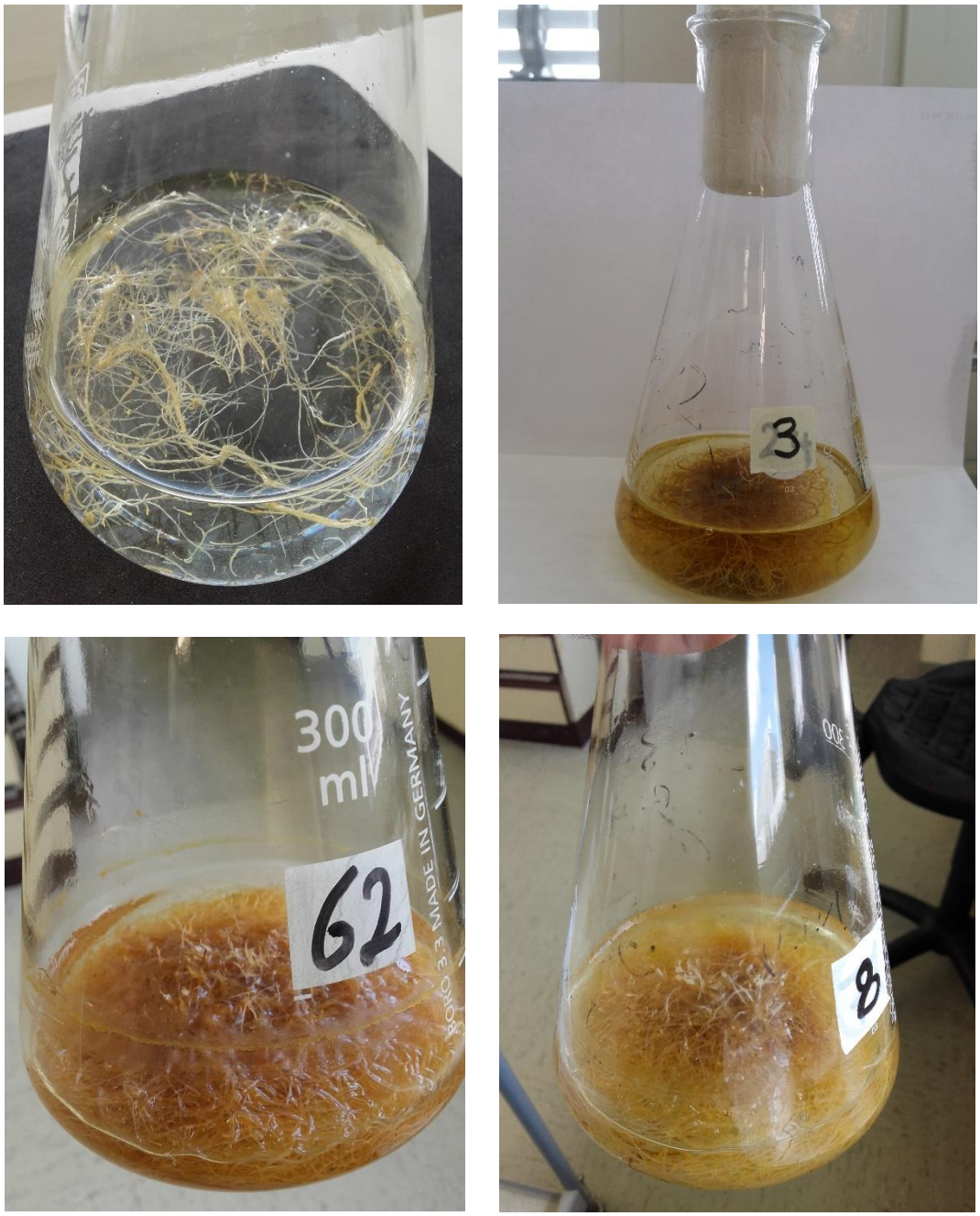

Figure 28: *Hyoscyamus niger* and *Sesamum indicum* hairy root in shaking flask culture

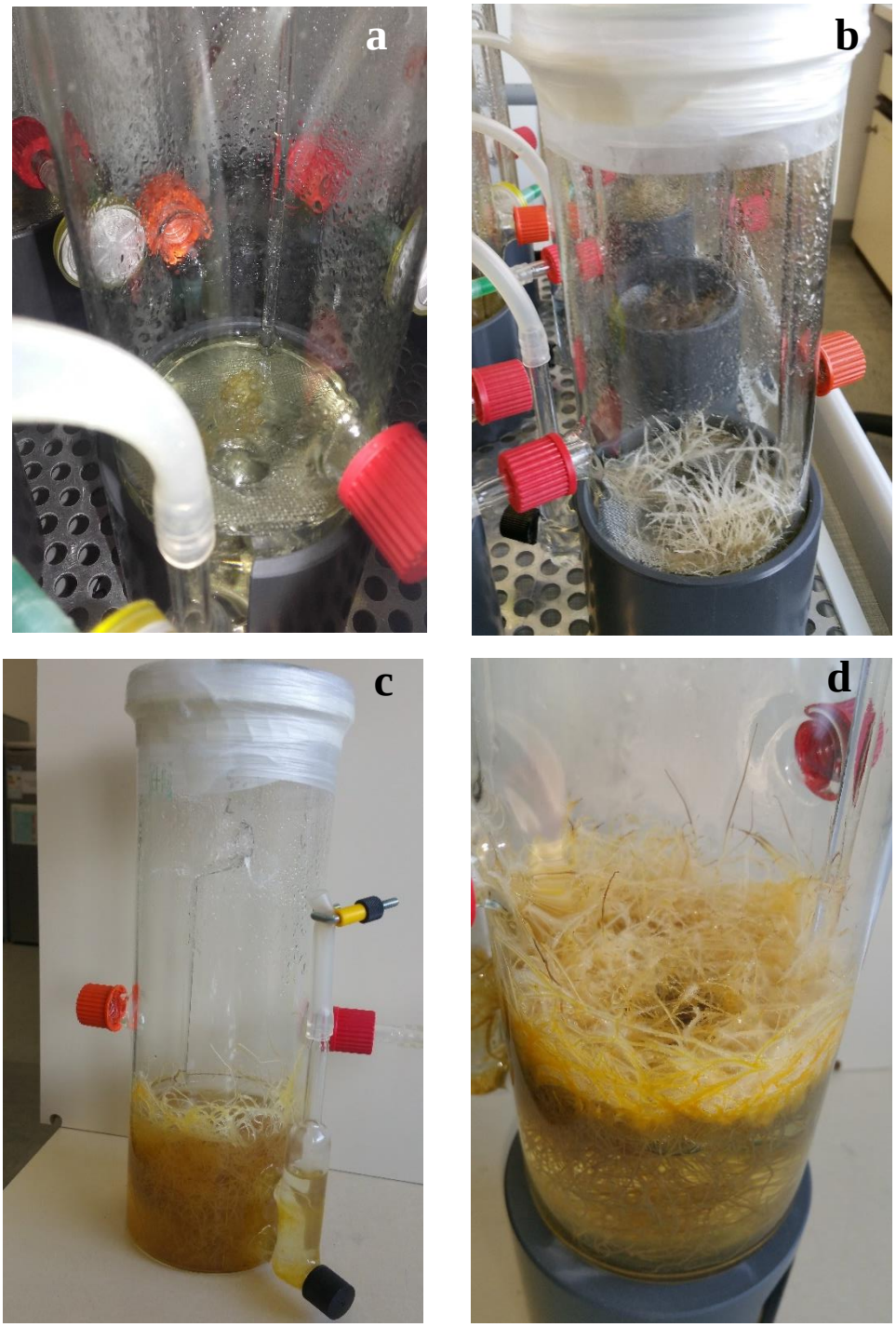

Figure 29: *Hyoscyamus niger* and *Sesamum indicum* hairy root, Bioreactor culture. From inoculation (a), and growing steeps (b, c, d)

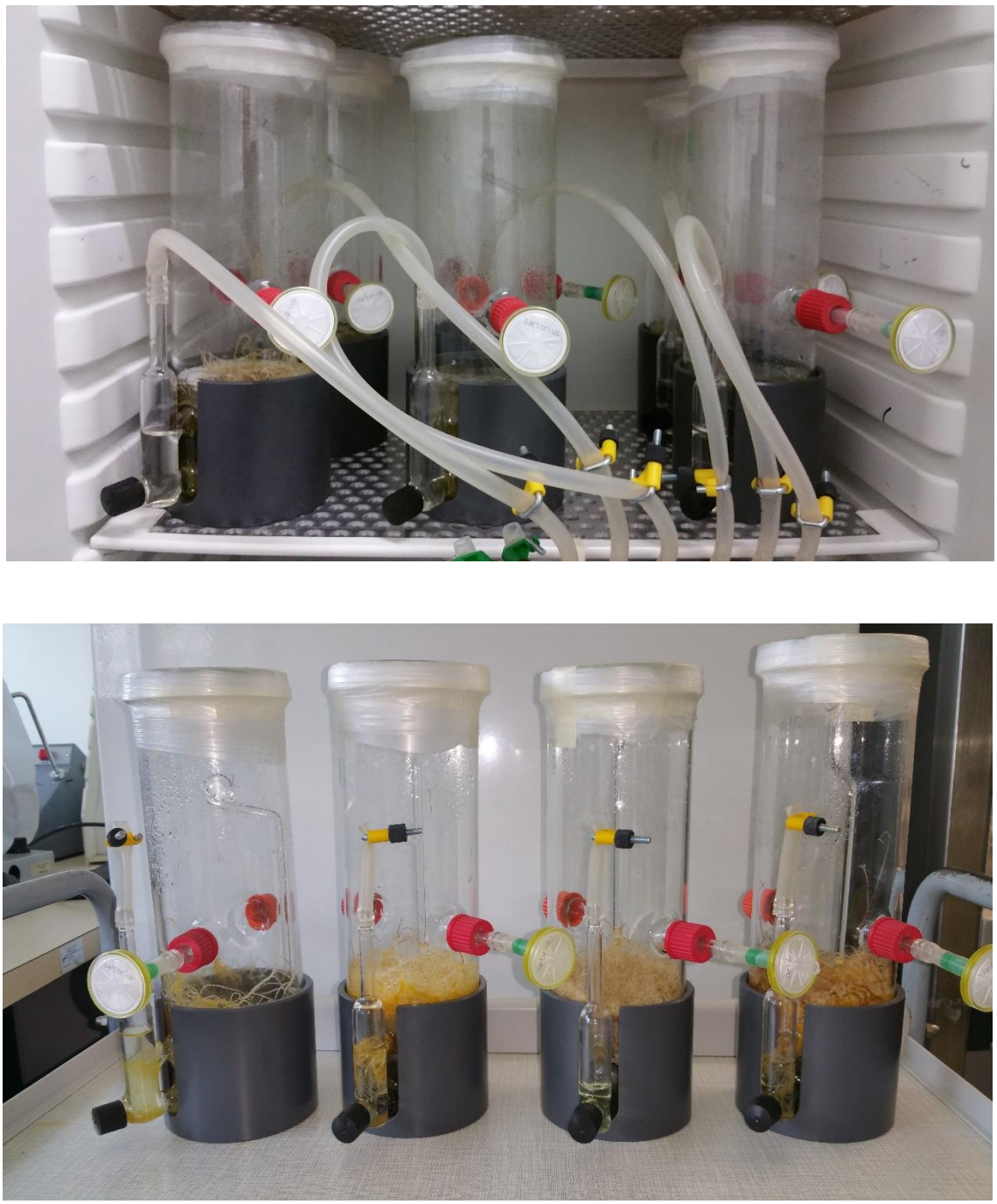

Figure 30: *Hyoscyamus niger* and *Sesamum indicum* hairy root Bioreactor culture

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7. CURRICULUM VITAE

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