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Nadja Förster

# Antikarzinogenes Potential ausgewählter Glucosinolate von *Moringa oleifera*

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# Antikarzinogenes Potential ausgewählter Glucosinolate von *Moringa oleifera*

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| *M. oleifera* | *Moringa oleifera* |
| MS-Medium | Murashige-Skoog-Medium |
| NQO1 | NAD(P)H:Chinonoxidoreduktase-1 |
| Nrf2 | Transkriptionsfaktor, Nuclear factor erythroid 2-related factor 2 |
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# Einleitung

*Moringa oleifera* Lam. (1785), auch als „Tree of Life“ bekannt und zu den Moringaceae gehörend, ist eine Pflanze mit vielfältigen Nutzungsmöglichkeiten. *M. oleifera* als bekannteste und am weitesten verbreitete von insgesamt 13 Arten (Antwi-Boasiako & Enninful, 2011) erlangte in den vergangenen Jahren in Form von Tees, Kosmetika und Nahrungsergänzungsmitteln weltweit immer mehr Bekanntheit. Die unterschiedlichen Pflanzenorgane von *M. oleifera* werden hierbei auf ganz verschiedene Art genutzt. So wird mikrobiell kontaminiertes Grundwasser in armen Regionen (u. a. Ägypten und Sudan) mit Hilfe von Pulver aus den Samen von *M. oleifera* kostengünstig auf Trinkwasserqualität gereinigt. *M. oleifera* dient hier als Coagulant (Foidl et al., 2001; Mangale Sapana et al., 2012). Auch besitzt das Öl der Samen, bekannt als „Ben Oil“, Eigenschaften volatile Stoffe zu binden und sehr stabil sowie langlebig zu sein (Foidl et al., 2001; Ramachandran et al., 1980). Daher wird es in der Kosmetik- und Parfümindustrie sowie als Schmiermittel in der Feinmechanik eingesetzt (Foidl et al., 2001; Ramachandran et al., 1980). Blattpulver von *M. oleifera* wird u. a. als Viehfutter verwendet (Kholif et al., 2015; Sultana et al., 2015), während die Rinde zur Herstellung von Seilen, Matten, Papier (Ramachandran et al., 1980) und Gerbstoffen (Mehta et al., 2011) dient. Aus den Blättern kann ein Extrakt generiert werden, welches u. a. Hormone des Cytokenintyps enthält, und daher wachstumsfördernd wirken können. Dieses kann höhere Erträge, mehr und größere Früchte, eine erhöhte Resistenz gegenüber Krankheiten und Krankheitserreger, höhere Keimungsraten und vieles mehr bei verschiedenen Pflanzenarten zur Folge haben (Foidl et al., 2001; Iqbal et al., 2015; Yasmeen et al., 2012). Besonders verbreitet ist jedoch die Verwendung von frischen Blättern von *M. oleifera* sowie Blattpulvern als Nahrungsmittel bzw. Nahrungsergänzungsmittel oder „Functional Food“ (Anwar et al., 2007). Die Blätter von *M. oleifera* stellen eine wichtige Proteinquelle dar. Weiterhin sind sehr hohe Gehalte an Kalium, Calcium, Eisen und vielen Vitaminen wie u. a. Provitamin A (ß-Carotin), C oder Vitamin E (α-Tocopherol) beschrieben (Foidl et al., 2001; Ramachandran et al., 1980; Yang et al. 2006). Da *M. oleifera* als trockenresistent gilt und seine Blätter erst spät in der Trockenzeit abwirft, dienen diese Blätter in einigen tropischen Ländern als einzige Quelle von Proteinen und Nährstoffen in der Trockenzeit. Aus diesem Grunde findet man den ursprünglich aus den Subhimalaya-

Gebieten von Nordindien, Pakistan, Bangladesch und Afghanistan stammenden Baum heute weitverbreitet in tropischen und subtropischen Gebieten, besonders in Ost- und Südafrika, sowie in Asien (Fahey, 2005; Fuglie & Sreeja, 2001). Hier wird *M. oleifera*, dessen Blätter mehrmals jährlich geerntet werden können, in Plantagen angebaut (u. a. Fuglie & Sreeja, 2001; Palada & Chang, 2003). Das frisch geerntete Pflanzenmaterial wird dann auf regionalen Märkten verkauft. Besonders in den afrikanischen Tropen spielt *M. oleifera* zur Bekämpfung von Hunger und insbesondere Mangelernährung eine entscheidende Rolle (Thurber & Fahey, 2009). So werden Blätter von *M. oleifera* schwangeren bzw. stillenden Frauen sowie Kindern in Form von Pulvern verabreicht. Darüber hinaus werden sie frisch verzehrt. Bei mangelernährten Kindern konnte der Ernährungs- und Gesundheitszustand hierdurch deutlich verbessert werden (signifikante Gewichtszunahme: Srikanth et al., 2014; bessere Gesundheit: Price, 2007). Weiterhin zeigten die Säuglinge direkt nach der Geburt ein höheres Gewicht und die Mütter produzierten mehr Milch zum Stillen der Kinder (Fuglie, 2001; Price, 2007).

Neben den genannten Verwendungsmöglichkeiten ist weiterhin eine vielseitige Einsetzbarkeit von *M. oleifera* als pflanzliches Arzneimittel hervorzuheben. Südafrikanische Stämme nutzen Extrakte, Sude, Breie, Öle, Salben und Pulver aus *M. oleifera*, um Entzündungen, Infektionskrankheiten, Fieber, Schwellungen, Kopfschmerzen, abdominale Tumore, Herz-Kreislauf-Beschwerden, Magen-Darm- und Nervenerkrankungen zu behandeln (reviewed in Mahmood et al., 2010; Sreelatha et al., 2011). Auf Basis dieses Stammeswissens und der mündlichen Überlieferungen von Wirksamkeiten begann eine Vielzahl an Autoren diese in wissenschaftlichen Studien zu analysieren (Anwar et al., 2007; reviewed in Fahey, 2005). U. a. konnten antibiotische, antimikrobielle (Cáceres et al., 1991; Eilert et al., 1981; Nikkon et al. 2003), antiinflammatorische (Cáceres et al., 1992; Ezeamuzie et al., 2008), antioxidative (Atawodi et al., 2010; Chumark et al., 2008; Kumar et al., 2012; Verma et al., 2009) und antikarzinogene (Bharali et al., 2003: Budda et al., 2011; Costa-Lotufo et al., 2005; Khalafalla et al., 2010; Parvathy & Umamakeskwari, 2007) Effekte von *M. oleifera*, meist in Gesamtextrakten der unterschiedlichen Pflanzenorgane, nachgewiesen werden. Diese Wirksamkeiten werden, wie auch schon bei anderen Pflanzenfamilien (z. B. Labiatae oder Zingiberaceae), auf die in der Pflanze vorkommenden sekundären Inhaltsstoffe zurückgeführt (reviewed in Craig, 1999). Sekundäre Pflanzeninhaltsstoffe

in *M. oleifera* sind Flavonoiden, Saponine, Phenolsäuren, Carotinoide und Glucosinolate (Förster et al., 2015a; Sharma & Paliwal, 2014; Shidduraju & Becker, 2003; Yang et al., 2006). Nur vereinzelt untersuchten Wissenschaftler in *in vitro*- und *in vivo*-Studien die genannten Effekte von einzelnen chemischen Verbindungen bzw. Inhaltsstoffgruppen von *M. oleifera* (Guevara et al., 1999; Murakami et al., 1998; Sharma & Paliwal, 2014). Die medizinische Wirksamkeit, im Besonderen das antioxidative und/oder antikarzinogene Potential, wird zum großen Teil auf die in allen Pflanzenteilen von *M. oleifera* enthaltenen Glucosinolate bzw. deren Hydrolyseprodukte zurückgeführt (Bennett et al., 2003). Eine Vielzahl an epidemiologischen Studien korrelierte eine erhöhte Aufnahme an Kohlgemüse mit einer protektiven Wirkung gegen Krebs. So erklärten Kirsh et al. (2007), dass das Risiko an Prostatakrebs zu erkranken mit der erhöhten Aufnahme von Kohlgemüse, speziell Brokkoli und Blumenkohl, reduziert werden kann. Verhoeven et al. (1996) verglichen die Ergebnisse von sieben Kohortenstudien und 87 Fall-Kontroll-Studien und stellten inverse Zusammenhänge zwischen dem Konsum von verschiedenen *Brassica*-Gemüsen und dem Krebsrisiko heraus. 67 % der Fall-Kontroll-Studien zeigten, dass sich ein erhöhter *Brassica*-Verzehr krebsreduzierend auswirkte (70, 56 und 67 % für Kohl, Brokkoli und Blumenkohl). Alle diese Studien führen die protektiven Effekte auf die in *Brassica* enthaltenen Glucosinolate, bzw. deren bioaktive Hydrolyseprodukte, zurück. So wurden dem Isothiocyanat von Glucoraphanin, Sulforaphan, welches in hohen Konzentrationen in Brokkoli nachzuweisen ist, chemoprotektive Effekte zugeschrieben (Review von Clarke et al., 2008). Die von Clarke et al. (2008) zitierten Studien untersuchten die Regulierung von Phase I- und Phase II-Enzymen und stellten fest, dass Sulforaphan die Krebs-Initiationsphase durch die Hemmung von Phase I-Enzymen und Stimulierung von Phase II-Enzymen blockieren kann. Auch Benzyl-Isothiocyanat bewirkte ein unterdrücktes Wachstum von Brustkrebszelllinien durch Zellzyklus-Arrest sowie Apoptoseinduktion (Xiao et al., 2006). *M. oleifera* besitzt im Vergleich zu anderen Pflanzen, welche Glucosinolate enthalten, sehr hohe Konzentrationen an diesen (Bennett et al., 2003; Förster et al. 2015a; Yang et al. 2006). Weiterhin ist ein zusätzlicher Zucker, die Rhamnose, am Benzolring des aromatischen Glucosinolates gebunden, welcher dem Molekül eine im Pflanzenreich sehr seltene Struktur verleiht.

Ein wichtiges Ziel vieler Forscher ist es, der Heilung bzw. Bekämpfung von Krebs näher zu kommen und die dahinter stehenden biologische Prozesse besser verstehen zu lernen. Dazu bedarf es der Aufklärung von Mechanismen und Abläufen der Krebsentstehung sowie der Identifizierung krebsfördernder und –hemmender Einflüsse. Sicherlich ist es möglich mit der Hilfe von Chemo- und Strahlentherapie als auch mit immer besser werdenden chirurgischen Techniken sowie mit einer zielgerichteten Therapie (z. B. mit Hilfe von monoklonalen Antikörpern) verschiedene Tumorarten mit hohen Erfolgsraten zu bekämpfen. Jedoch gilt Krebs noch immer als häufigste Todesursache auf der Welt (8,2 Millionen krebsbedingte Sterbefälle 2012; Stewart & Wild, 2014). Weltweit wurden 2012 14 Millionen neue Krebserkrankungen diagnostiziert, 3,2 Millionen davon allein in Europa (Torre et al., 2015). Im Jahre 2012 wurde bei 477.950 Deutschen Krebs diagnostiziert, 2016 werden schätzungsweise eine halbe Million Menschen in Deutschland neu an Krebs erkranken (RKI & GEKID, 2015). Auch wenn synthetische Arzneimittel als wirksamer gelten, stoßen Phytopharmaka auf eine immer größere Akzeptanz in unserer Gesellschaft. So betrug der Anteil der Phytopharmaka im Jahr 2014 21 % am Umsatz mit rezeptfreien Arzneimitteln im Apothekenmarkt (BAH, 2015). Wie am Beispiel von Acetylsalicylsäure gelten sie häufig als verträglicher, da die sekundären Pflanzeninhaltsstoffe in eine organische Umgebung eingebettet sind und maßvoll dosiert werden. Weiterhin sind in einem pflanzlichen Arzneimittel meist mehrere Wirkstoffe vereint. Noch heute sind Pflanzen, welche in traditioneller sowie Stammesmedizin aufgrund verschiedener potentiell heilender Effekte genutzt wurden und werden, daher Forschungsgegenstand. So weist auch *M. oleifera* ein hohes bioaktives Potential auf, von dem man noch nicht genau klären konnte, auf welche Inhaltsstoffe dieses zurückgeführt werden kann. Die vorliegende Dissertation soll daher einen Beitrag dazu leisten das antioxidative und antikarzinogene Potential der Glucosinolate von *M. oleifera* besser einschätzen zu können. Bedingt durch die aufeinander aufbauenden Fragestellungen ergab sich folgende Struktur der Arbeit:

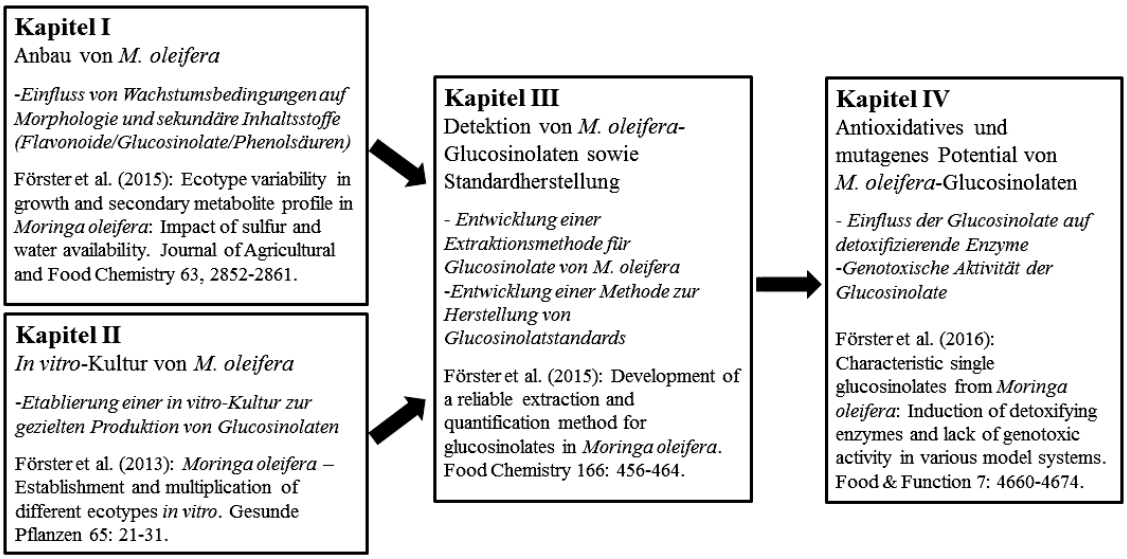



Abb. 1: Aufbau der vorliegenden Dissertation

Mit Hilfe eines gezielten Anbaus sollte im Gewächshaus (Kapitel I) sowie durch *in vitro*-Kultur (Kapitel II) Blattmaterial von *M. oleifera* produziert werden, welches ausreichende Mengen an Glucosinolaten beinhaltete. Die Blätter dienten als Grundlage für die Entwicklung eines geeigneten Extraktionsverfahrens dieser sekundären Inhaltsstoffe (Kapitel III). Stabile Glucosinolatstandards (Gesamtextrakt sowie Einzelsubstanzen) wurden hergestellt (Kapitel III), um im Folgenden potentielle medizinische Wirksamkeiten dieser aus *M. oleifera* isolierten Substanzen zu untersuchen (Kapitel IV).

## Kapitel I - Anbau von *M. oleifera*

*M. oleifera* weist im Vergleich zu anderen Arten der Gattung *Moringa* enorm hohe Glucosinolatgehalte auf (Bellostas et al., 2010). Neben Diversitäten im Inhaltsstoffprofil konnten auch Unterschiede im Wuchsverhalten oder in der Blattmasseproduktion festgestellt werden (Doerr et al., 2009; Palada & Chang, 2003; eigene Beobachtungen). Diese waren nicht nur interspezifisch (zwischen verschiedenen Arten) sondern auch intraspezifisch (innerhalb einer Art) nachzuweisen. Im Intensivanbau von *M. oleifera* wurden stark wachsende Ökotypen verwendet, das Inhaltsstoffspektrum spielte bis zum heutigen Zeitpunkt eine untergeordnete/keine Rolle (Palada & Chang, 2003). Variabilitäten können zum einen genetisch begründet sein (Ökotypen) und/oder aufgrund von unterschiedlichen Wachstumsbedingungen in den Herkunftsstandorten zustande kommen (Herkünfte).

Neben der genetischen Konstitution können auch Anbaubedingungen das Wachstum sowie die Inhaltsstoffgehalte von Pflanzen, so auch *M. oleifera*, beeinflussen. Eine Vielzahl an Studien an Brassicaceen (glucosinolathaltige Pflanzen) bewies den Einfluss von Düngung auf den Glucosinolatgehalt unterschiedlichster Pflanzenorgane. Aromatische Glucosinolate, wie sie in den Blättern von *M. oleifera* vorzufinden sind, befinden sich jedoch besonders in der Wurzel oder in den Samen von Pflanzen (Bennett et al., 2004; Dam et al., 2009). Düngungsstudien über die Beeinflussung von aromatischen Glucosinolaten in Blättern sind daher rar bzw. fokussieren meist auf die aliphatischen sowie indolischen Vertreter dieser sekundären Inhaltsstoffe. Bloem et al. (2007) zeigten jedoch, dass eine erhöhte Schwefeldüngung den Glucotropaeolingehalt in den Blättern der Kapuzinerkresse um das 1,4 – 2,1-fache erhöhte. Eine erhöhte Stickstoffdüngung beeinflusste den Glucotropaeolingehalt nicht. Untersuchungen von Kopsell et al. (2007) an Wasserkresse stellten die Stickstoff- sowie Schwefeldüngung als beeinflussende Parameter für den Gehalt an aromatischen Glucosinolaten heraus. Die Autoren detektierten in Wasserkresse Glucosinalbin und Gluconasturtiin als aromatische Glucosinolate. Auch Trockenstress kann sekundäre Pflanzeninhaltsstoffe in unterschiedlichem Maße beeinflussen. So konnten Zhang et al. (2008) zeigen, dass die Konzentration von aromatischen Glucosinolaten in der Wurzel von *Brassica rapa* ssp. *rapifera* L. (Rübe) abhängig von der Anbauzeit durch eine unterschiedliche

Wasserversorgung variieren konnte. Auch wurde der Glucosinolatgehalt des Rapssamens durch Trockenstress zu unterschiedlichen Pflanzenentwicklungszeiten positiv oder negativ beeinflusst (Bouchereau et al., 1996). Bloem et al. (2014) veröffentlichten eine Studie, in der sie zeigten, dass Trockenstress die Glucotropaeolinkonzentration in Kapuzinerkresse erhöhte, die Biomasse sank jedoch gleichzeitig ab.

Aufgrund der Tatsache, dass verschiedene Herkünfte von *M. oleifera* phänotypische Unterschiede zeigten, der Baum generell als trockenresistent gilt (Anbau in Tropen und Subtropen) und enorm hohe Gehalte an Glucosinolaten (schwefelhaltigen Verbindungen) sowie hohe Gehalte an Flavonoiden (UV-Pigmente) aufweist, sollten folgende Sachverhalte untersucht werden:

- *Unterscheiden sich die Ökotypen/Herkünfte morphologisch phänotypisch (Biomasse, Höhe)/oder chemisch phänotypisch (Gehalt und Spektrum an sekundären Inhaltsstoffen)bei gleichen Wachstumsbedingungen?*
- *Können wir von der Existenz verschiedener M. oleifera-Ökotypen ausgehen oder handelt es sich um unterschiedliche Herkünfte?*
- *Ist ein Trade off zwischen Wachstum und Inhaltsstoffproduktion zu beobachten?*
- *Kann man einen „Top"-Ökotypen identifizieren, welcher eine hohe Biomasse und hohe Gehalte an Sekundärmetaboliten vereint?*
- *Wie wirken sich Trockenheit und Schwefeldüngung auf das Pflanzenwachstum sowie die sekundären Inhaltsstoffe (Glucosinolate und Flavonoide) von M. oleifera aus? Wie reagieren die verschiedenen Ökotypen?*
- *Kann man Rückschlüsse auf optimale Anbaubedingungen für M. oleifera ziehen?*

Die in diesem Kapitel dargestellten Fragestellungen werden in folgender Publikation behandelt:

Förster, N.; Ulrichs, C.; Schreiner, M.; Arndt, N.; Schmidt, R. & Mewis, I. (2015): Ecotype variability in growth and secondary metabolite profile in *Moringa oleifera*: Impact of sulfur and water availability. *Journal of Agricultural and Food Chemistry* 63: 2852-2861.

## Abstract

*Moringa oleifera* is widely cultivated in plantations in the tropics and subtropics. Previous cultivation studies with *M. oleifera* focused primarily only on leaf yield. In the present study the contents of potentially health-promoting secondary metabolites, (glucosinolates, phenolic acids, and flavonoids) were also investigated. Six different ecotypes were grown under similar environmental conditions to identify phenotypic differences that can be traced back to the genotype. The ecotypes TOT4880 (origin USA) and TOT7267 (origin India) were identified as having the best growth performance and highest secondary metabolite production, making them an ideal health-promoting food crop. Furthermore, optimal cultivation conditions - exemplarily on sulfur fertilization and water availability - for achieving high leaf and secondary metabolite yields were investigated for *M. oleifera*. In general, plant biomass and height decreased under water deficiency compared to normal cultivation conditions, while the glucosinolate content increased. The effects depended to a great extent on the ecotype.

## Introduction

*Moringa oleifera* Lam., the main representative of the order *Moringa* and originally native to the sub-Himalayan region, is commonly grown on plantations in Asia and Africa (Fuglie et al., 2001). All plant parts of *M. oleifera* are edible and contain a multitude of nutrients such as high levels of essential amino acids, iron, calcium, and carotenoids (Ferreira et al., 2008). *M. oleifera* can be cultivated in different ways, but more often the tree is cultivated intensively in plantations to harvest large quantities of leaf material (Fuglie et al., 2001; Gamatie, 2001; Olivier; Palada, 1995; Palada & Chang, 2003; Radovich). Especially in Asia and Africa this tree is cultivated commercially (Bellostas et al., 2010; Fahey, 2005; Gamatie, 2001; Radovich; Saha et al., 2012). Bellostas et al. (2010) reported that *Moringa* leaves rank first among the most widely consumed leafy vegetables in Niger. *M. oleifera* leaves provide a good basis for satisfying the population's nutritional needs. Additionally, *M. oleifera* is known to be drought resistant and is therefore an important source of food during the dry season, especially in the African tropics (Thurber & Fahey, 2009).

Besides its high nutritional potential, *M. oleifera* has been known in Asia and Africa for centuries as a traditional remedy. Antimicrobial, anti-inflammatory, detoxifying, and anticancerogenic effects of *M. oleifera* were described among other effects in literature (reviewed in Fahey, 2005). These health-promoting effects have largely been attributed to glucosinolates or rather their biological active hydrolysis products.

It is known that *M. oleifera* ecotypes show a diverse growth performance and leaf mass production. *M. oleifera* leaves are consumed because of their high nutritional value. The high levels of potential health-promoting secondary metabolites, especially the glucosinolates, have received less attention in cultivation experiments. However, high glucosinolate levels might not be present in ecotypes with a high leaf mass productivity. Therefore, in the present study ecotypes of *M. oleifera* with a strong differing growth performance were used to investigate the possible trade-offs between biomass and glucosinolate accumulation to determine the overall best yielding ecotypes. Six ecotypes were used to determine to what extent ecotypes show morphological (height, biomass) and chemical (secondary metabolite content and composition) phenotypic differences.

In addition to glucosinolates, *M. oleifera* leaves also exhibit high contents of phenolic acids and flavonoids (Bennett et al, 2003; Siddhuraju & Becker, 2003). Different authors relate health-promoting effects of phenolics to their high antioxidative potential (reviewed in Dai & Mumper, 2010). For this reason, phenolic acids and flavonoids were also analyzed in this study, although the focus remained on glucosinolates.

Cultivation conditions can influence plant growth and secondary metabolite contents. Different authors have shown in their analyses the influence of fertilization on the growth and glucosinolate content of brassicaceous plants (Ahmad et al., 2007; Aires et al., 2006; Bloem et al., 2007). Aromatic glucosinolates represent only a small chemical group in glucosinolate containing brassicaceous vegetable leaves and were rarely studied in cultivation experiments. One study analyzing the influence of fertilization on the leaf glucosinolates of *Tropaeolum majus* L. showed that sulfur fertilization increased the benzyl glucosinolate content, whereas nitrogen fertilization had no significant influence (Bloem et al., 2007). *M. oleifera* contained in all plant parts (below- and above-ground) very high contents of aromatic glucosinolates, which is a characteristic that makes this plant unique. In addition to some other trees in the families of Akaniaceae,

Bretschneideraceae, or Gyrostemonaceae (Mithen et al., 2010), *M. oleifera* has in all plant parts considerable quantities of glucosinolates. Our knowledge of the glucosinolate content in tree species is only rudimentary compared to the numerous studies that have been conducted on the influence of nitrogen and/or sulfur fertilization on brassicaceous leafy plants.

Several studies have been undertaken about nitrogen and sulfur fertilization and their influence on phenolics in Brassicaceae (Li et al., 2008; Pascale et al., 2007; Sousa et al., 2008; Vallejo et al., 2003). Li and colleagues (2008) as well as other different authors (Pascale et al., 2007; Vallejo et al., 2003) found an improving influence of sulfur fertilization on phenolic compounds in different *Brassica* species. Based on the findings of the aforementioned studies, the influence of sulfur fertilization on the content and composition of phenolic acids and flavonoids was analyzed using *M. oleifera* ecotypes.

Because of the fact that one characteristic of *M. oleifera* is drought tolerance and harvest takes place all year round, the influence of water deficiency on the poly-glycosylated aromatic glucosinolates was also analyzed. Because *M. oleifera* is not an annual plant like other herbaceous species containing glucosinolate, results are difficult to predict. Analysis of other authors showed enhancing or decreasing effects depending on the extent of water stress (e. g. Khan et al., 2010; Zhang et al., 2008) and plant species (e. g. Dam et al., 2009; Halkier & Gershenzon, 2006). On the basis of different previous studies, which show increasing phenolic content after drought stress (Abreu & Mazzafera, 2005; Hernández et al., 2004), a change in the content and composition of phenolics in *M. oleifera* leaves is conceivable. Therefore, the corresponding analyses were included in the present study.

Because studies on accumulation of secondary metabolites under varying conditions are rare or nonexistent for *M. oleifera*, effects of sulfur fertilization and drought stress on plant growth and levels of secondary metabolites in the leaves of the different *M. oleifera* ecotypes were investigated. The results will generate information providing insights into an optimal cultivation of *M. oleifera* ecotypes. The overarching aim of the study was to find differences in *M. oleifera* ecotypes in growth and secondary metabolite content to determine the most suitable ecotype for leaf and secondary metabolite production. The goal was to identify an optimal cultivation variant with the best plant growth performance

and the highest secondary metabolites content. The results provided information about how to achieve a high yield in biomass as well as potentially health-promoting ingredients in the cultivation of *M. oleifera*.

## Materials and methods

### Cultivation experiments in Großbeeren

After a prescreening using 13 different ecotypes of *Moringa oleifera* Lam., seeds of the six best germinating and -growing ecotypes were selected for the experiments. The *M. oleifera* ecotypes were precultivated in a greenhouse at the Leibniz-Institute of Vegetable and Ornamental Crops in Großbeeren (Germany). Seeds from four ecotypes adapted to different climatic conditions were obtained from the AVRDC - The World Vegetable Center (Taiwan): TOT4880 (origin USA; ecotype 1), TOT5028, TOT7277 (origin Thailand; ecotypes 2 and 3), TOT7267 (origin India; ecotype 4). Seeds from the other two ecotypes were collected in the Philippines and Taiwan (ecotypes 5 and 6). Seedlings (eight weeks old) were planted in grid patterns at 0.5 m apart in a 1 m deep enclosed greenhouse bed. Drip irrigation and a nebulization system were used to humidify soil and air. In 2010, the mean temperature during the vegetation period (from June to October) in the greenhouse was 19.5 °C (2011: 18.6 °C, 2012: 19.5 °C) and varied during this period between a maximum temperature of 23.5 °C in July and a minimum temperature of 16.3 °C in October (2011: 20.5 °C in August and 14.6 °C in October, 2012: 22.3 °C in July and 15.7 °C in October). The ventilation temperature was set to 19 °C. Biomass production, height, and total glucosinolate content were determined to analyze ecotype differences. Plant samples were taken in August 2010 (first harvest, H1); November 2010 (second harvest, H2); July 2011 (third harvest, H3); October 2011 (fourth harvest, H4) and August 2012 (fifth harvest, H5). The leaf/stem ratio of well-established plants was analyzed from plant material at the second harvest, November 2010. By this time, the plants had grown into completely developed trees.

Furthermore, the influence of different growing variants on the growth and secondary metabolite contents (glucosinolates, phenolic acids, and flavonoids) of the six *M. oleifera* ecotypes was analyzed in November 2010 (second harvest) and in July 2011 (third harvest) (normal variant, sulfur variant, water deficiency variant, Suppl. Table 1). Per

variant and ecotype five plants were cultivated. Each growing variant was located in a closed bed surrounded with concrete, separating the variants from each other. All beds were fertilized (basic fertilization) with 7.04 g/m$^2$ nitrogen (372 g calcium ammonium nitrate per parcel, twice a year), 2.56 g/m$^2$ phosphorus (Superphospate, 465 g phosphorus pent-oxide per parcel), and 5.76 g/m$^2$ potassium (Patentkali®, 330 g potassium oxide per parcel) once a year at the end of April/beginning of May. The plants of the sulfur variant received an additional 0.704 g/m$^2$ sulfur annually (56 g potassium sulfate per parcel). To reach low to moderate water deficiency in one bed, the irrigation and the nebulization system for the whole cabin was switched off in defined periods (August – November 2010, June – September 2011). In the cultivation beds containing the normal and sulfur variant, a continuous soil water tension of approximately 50 – 100 hPa was maintained. In the bed with the water deficiency variant, a higher soil water tension was determined (second harvest: 380 hPa, third harvest: 175 hPa; Suppl. Figure 1). At the second harvest, the upper limit of the field capacity was exceeded (FC ranged between 63 – 316 hPa, therefore pF = 1.8 - 2.5). The water content of the sandy soil was at this time < 2%. At the third point of harvest, the soil water tension was in the range of field capacity. Tensiometers used had a measurement range between 0 and 1.000 hPa. A rewetting of the dry bed from December 2010 to May 2011 was necessary to prevent plants from death. Wilting and chlorosis of leaves have been recognized.

Leaves from the top of the plants (upper 20 cm, youngest leaves) were used for the analysis of glucosinolates, flavonoids, and phenolic acids (secondary metabolites). In this way, it was possible to guarantee that leaf material of the same age was used for analysis. The glucosinolate content of older and middle-aged leaves was determined additionally to get an idea of the allocation of the glucosinolate over the whole plant. After each sampling, the whole plantation was pruned back to a height of 60 – 70 cm. Because of space limitations in the greenhouse, a doubled pruning of the plants was required. The biomass was determined at each harvest by weighing the fresh cut plant material (leaves and stem of each plant, which exceeded the 60 – 70 cm). As an example, for the first harvest, the glucosinolate profit per plant was calculated to show ecotype variability (Suppl. Table 2).

For all cultivation variants, samples from five different plants per ecotype were taken according to Figure 1. Therefore, the analysis was performed in five replications.

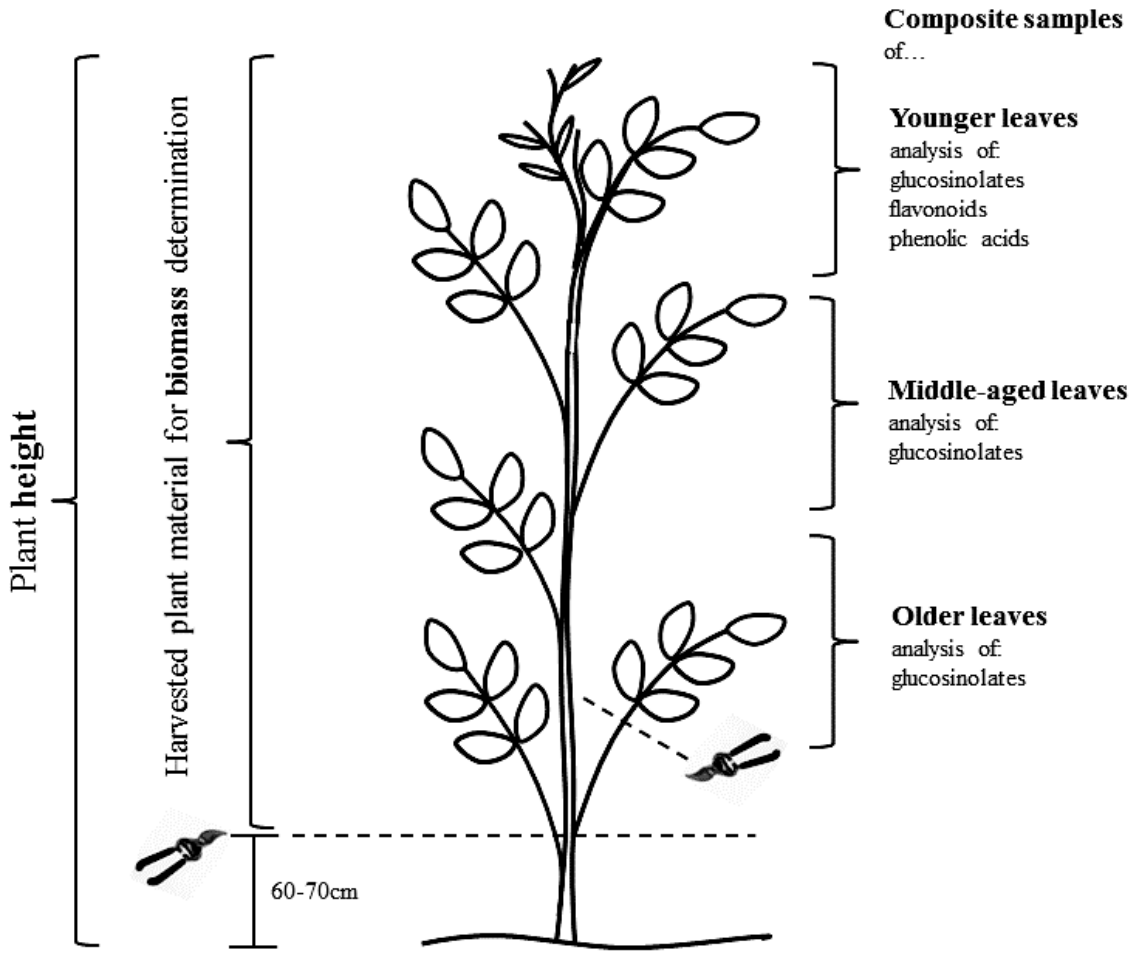



Fig. 1: Sampling of plant material of *Moringa oleifera*

**Determination of glucosinolates**

Leaf material for glucosinolate analysis was shock-frozen in liquid nitrogen and kept at -20 °C until freeze-drying. The content and composition of *M. oleifera* glucosinolates were determined using a method for extraction of intact glucosinolates described in detail by Förster et al. (2015). Briefly, a methanolic extraction (70%) was performed in three steps with 20 mg of pulverized leaf material (Retsch Mixer Mill MM 301, fineness about 5 µm, manufacturer specifications). The combined supernatant was concentrated (vacuum concentrator, Thermo Scientific Savent SPD111V Concentrator, vacuum pump: Vacuumbrand PC 3000 series, SVC3000) and precipitated with barium acetate (0.4 M).

After precipitation (30 min) samples were centrifuged (16,000 g, Thermo Scientific, Heraeus Megafuge 11R Centrifuge for 10 min), decanted, and filled up to 2 ml with ultra-pure water. In each HPLC vial, 1 ml filtered sample (Costar® SpinX tubes) was transferred. The remaining amount of each sample was incubated with 0.05 U myrosinase (thioglucosidase, Sigma Aldrich) for 8 h at 37 °C to hydrolyze glucosinolates to their corresponding breakdown products. Hydrolyzed samples were filtered (Costar® SpinX tube) and transferred to HPLC sample vials for analysis. The internal standard, 2-propenyl glucosinolate, was added at the first extraction step.

Extracts were qualitatively and quantitatively analyzed on a Merck Hitachi HPLC system. A volume of 10 µl sample was injected (LaChrom L-7200 auto sampler) and separated on a 4.6 x 250 mm SB-C18 column (Zorbax 5 µm, Agilent) using a gradient program described in Förster et al. (2015). Two solvents were used for the HPLC analysis: solvent A, 100% 0.1 M ammonium acetate, solvent B, 40% acetonitrile/0.1 M ammonium acetate. Detection was performed at 229 nm using a photodiode array detector (LaChrom L-7455), and components were identified from the retention time and quantified against internal standard. The peak area remaining after myrosinase treatment of the intact glucosinolate extract was subtracted from the peak area of the glucosinolate of the intact glucosinolate extract without myrosinase treatment. Interfering/co-eluting ballast compounds were excluded from the calculation by using myrosinase (detailed described in Förster et al., 2015). Relative response factors (RF) were used according to Förster et al. (2015) and were therefore generated from fractionated standards.

**Determination of flavonoids and phenolic acids**

Flavonoids and phenolic acids in *M. oleifera* leaves were analyzed using a slightly modified HPLC method described in Mewis et al. (2011). A total of 20 mg lyophilized, powdered leaf material was extracted in 300 µl 70% methanol (pH 4, acetic acid) for 15 min in ice water using sonification (Bandelin Sonorex Super AG 102H). The pellet was re-extracted twice with 300 µl of the extraction solvent for 10 min. After each extraction step the samples were centrifuged for 5 min at 16,000 g (Thermo Scientific, Heraeus Megafuge 11R Centrifuge), and the supernatants were combined. Supernatants were concentrated (vacuum concentrator, Thermo Scientific Savent SPD111V Concentrator, vacuum pump: Vacuumbrand PC 3000 series, SVC3000) to near dryness,

dissolved in 50% methanol, and filled up to 1 ml. The samples were centrifuged for 2 min, filtered (Costar® SpinX tubes), and transferred to HPLC vials. 4-Methoxycinnamic acid (1 mmol/l, Sigma Aldrich) was used as internal standard.

Extracts were qualitatively and quantitatively analyzed on a HPLC-MS/MS system (Agilent 1100 Series, Waldbronn, Germany). A volume of 10 µl extract was injected and separated using a 150 x 2.1mm C16 column (AcclaimPA, 3 µm, Dionex) with the following gradient program: 0 - 1 min, 0.5% B; 1 - 10 min, 0.5 - 40% B; 10 - 12 min, 40% B; 12 - 18 min, 40 - 80% B; 18 - 20 min, 80% B; 20 - 24 min, 80 - 100% B; 24 - 30 min, 100% B; 30 - 34 min, 100 - 0.5% B; and 34 - 39 min, 0.5% B at a flow rate of 0.4 ml/min. Two solvents were used for analysis: solvent A, $H_2O$ (0.5% formic acid), and solvent B, 40% acetonitrile. The oven temperature was 35 °C. Detection was carried out at 290 nm, 320 nm and 370 nm on a photodiode array detector. Because all components showed a good signal at 290 nm, the compounds were quantified against internal standard 4-methoxycinnamic acid detected at this wave length. Compounds were identified as deprotonated molecular ions and characteristic mass fragment ions by HPLC-DAD-ESI-$MS^2$. Commercially available standards of single compounds were used as reference. Relative response factors (RF) were used to correct for absorbance differences between the reference standard 4-methoxycinnamic acid (RF = 1): RF = 1.16 for chlorogenic acid (Sigma Aldrich) and an isomer of chlorogenic acid, RF = 1.06 for neochlorogenic acid (Sigma Aldrich), RF = 1 for the two isomers of *p*-coumaroyl quinic acid and an isomer of feruloyl quinic acid (commercial standard not available), RF = 1.51 for vitexin (Fluka) and isovitexin, RF = 1.53 for quercetin 3-*O*-glucoside (Carl Roth GmbH), RF = 1.4 for kaempferol 3-*O*-glucoside (Carl Roth GmbH), RF = 1.84 for quercetin 3-*O*-(6´´-malonylglucoside) (Sigma Aldrich) and kaempferol 3-*O*-(6´´-malonylglucoside) (commercial standard not available).

**Statistical analysis**

Data were analyzed for significant differences using analysis of variance (ANOVA) following the mean comparison test, Tukey's HSD, with SigmaPlot 12.0. Cross dependence was analyzed by using the two-way ANOVA. The p-value was fixed at $p < 0.05$; any exceptions are mentioned in the text.

# Results

## Ecotype differences

*Biomass, height, glucosinolate content*

In addition to the growth parameters, ecotype differences in secondary metabolites were analyzed by focusing on the glucosinolates that were considered to have health-promoting effects. The data were collected at five different harvest points (Table 1). A very high variability among ecotypes replicates in biomass production, height, and glucosinolate content can be noticed. Significant differences were hard to identify. When considering all points of harvest, ecotypes 1, 2, and 4 had the highest biomass. Ecotypes 2, 3, 5, and 6 were significantly smaller than ecotype 4 (Table 1). On average across all ecotypes at each point of harvest, the biomass production and plant height were significantly higher at the third and fifth points of harvest in comparison to the other points of harvest. Considering the glucosinolate levels, ecotype 3 contained significantly lower glucosinolate content than the other ecotypes (Table 1). Ecotype 1 showed the highest glucosinolate content overall. The mean glucosinolate content of all analyzed ecotypes was significantly lower at the first point of harvest. At the second and fifth points of harvest, the ecotypes showed a significantly higher glucosinolate content in comparison to the other points of harvest. No significant interaction between the point of harvest and the ecotype was found for the biomass production, plant height, and glucosinolate content (two-way ANOVA, $p < 0.05$). Taking the height, biomass, and glucosinolate content of the five harvest points into consideration, ecotypes 1 and 4 were found to be the best performers in terms of biomass production and glucosinolate amounts in leaves. Not surprisingly, we detected a correlation between height and biomass. With an elimination of extreme values, a polynomial dependency ($y = 0.0153x^2 - 0.7184x$, $R^2 = 0.80$) was found. In contrast, no correlation was found between biomass/height and glucosinolate content. Also, the leaf-stem ratio did not differ between the six *M. oleifera* ecotypes. A total of 60% of the tree biomass harvested was formed by the leaves (Suppl. Table 2).

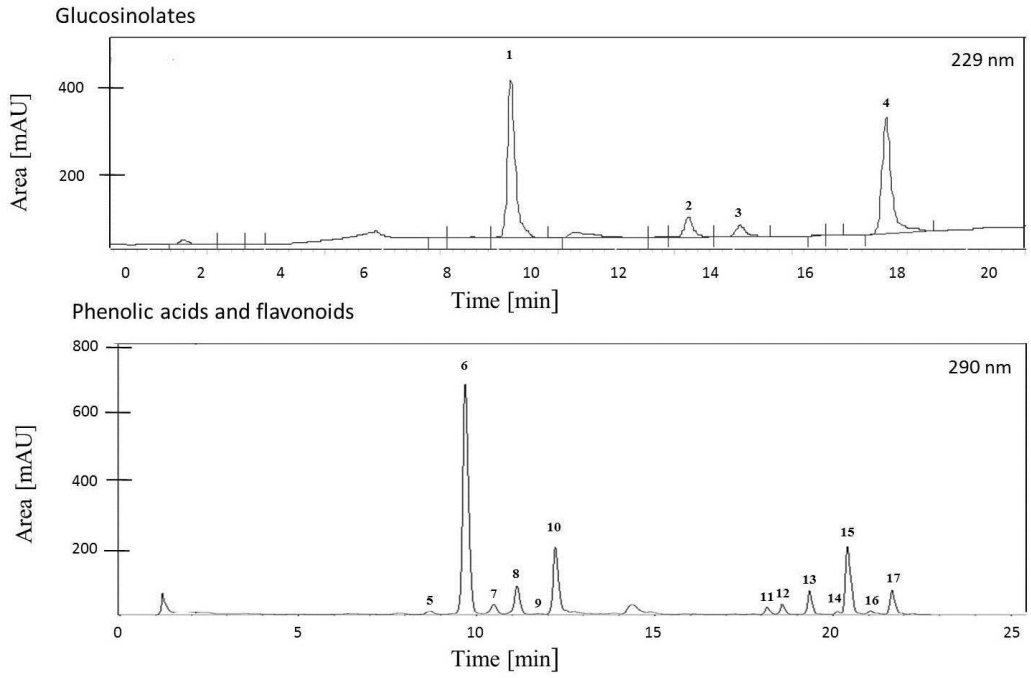
Tab. 1: Biomass, height, and total glucosinolate content of six different *Moringa oleifera* ecotypes at five harvest times

| **Biomass [g fw]** | **Ecotype** | | | | | | |
|---|---|---|---|---|---|---|---|
| **Harvest** | **E1** | **E2** | **E3** | **E4** | **E5** | **E6** | $\bar{x}$ |
| **H1** | $104_{(B)}$ | $253_{(B)}$ | 122 | 331 | 154 | 94 | **176$_{(B)}$** |
| **H2** | $416_{(AB)}$ | $270_{(B)}$ | 269 | 353 | 228 | 257 | **272$_{(B)}$** |
| **H3** | $883_{(ab,A)}$ | $1137_{(a,A)}$ | $385_{(b)}$ | $626_{(ab)}$ | $286_{(b)}$ | $301_{(b)}$ | **603$_{(A)}$** |
| **H4** | $443_{(AB)}$ | $340_{(B)}$ | 161 | 463 | 175 | 178 | **293$_{(B)}$** |
| **H5** | $769_{(AB)}$ | $468_{(AB)}$ | 661 | 844 | 451 | 437 | **605$_{(A)}$** |
| $\bar{x}$ | **523** | **494** | **320** | **523** | **259** | **253** | |
| **Height [cm]** | **Ecotype** | | | | | | |
| **Harvest** | **E1** | **E2** | **E3** | **E4** | **E5** | **E6** | $\bar{x}$ |
| **H1** | $140_{(B)}$ | $135_{(AB)}$ | 111 | 176 | 151 | 117 | **138$_{(B)}$** |
| **H2** | $166_{(AB)}$ | $122_{(B)}$ | 163 | 195 | 151 | 164 | **155$_{(B)}$** |
| **H3** | $215_{(ab,AB)}$ | $216_{(ab,A)}$ | $183_{(b)}$ | $260_{(a)}$ | $164_{(ab)}$ | $176_{(ab)}$ | **187$_{(A)}$** |
| **H4** | $153_{(ab,B)}$ | $145_{(ab,AB)}$ | $103_{(b)}$ | $201_{(a)}$ | $101_{(b)}$ | $102_{(b)}$ | **134$_{(B)}$** |
| **H5** | $226_{(A)}$ | $171_{(AB)}$ | 172 | 219 | 162 | 171 | **187$_{(A)}$** |
| $\bar{x}$ | **175$_{(ab)}$** | **153$_{(bc)}$** | **138$_{(c)}$** | **206$_{(a)}$** | **146$_{(bc)}$** | **144$_{(bc)}$** | |
| **GS$^{z}$ [µmol/g dw]** | **Ecotype** | | | | | | |
| **Harvest** | **E1** | **E2** | **E3** | **E4** | **E5** | **E6** | $\bar{x}$ |
| **H1** | $148_{(B)}$ | $155_{(B)}$ | $101_{(B)}$ | $144_{(B)}$ | $129_{(B)}$ | $110_{(B)}$ | **131$_{(C)}$** |
| **H2** | $348_{(a,A)}$ | $267_{(ab,A)}$ | $237_{(b,A)}$ | $285_{(ab,A)}$ | $274_{(ab,A)}$ | $266_{(ab,A)}$ | **276$_{(A)}$** |
| **H3** | $194_{(B)}$ | $202_{(AB)}$ | $180_{(AB)}$ | $214_{(AB)}$ | $141_{(AB)}$ | $119_{(B)}$ | **175$_{(B)}$** |
| **H4** | $200_{(a,B)}$ | $194_{(a,AB)}$ | $68_{(b,C)}$ | $217_{(a,AB)}$ | $169_{(a,AB)}$ | $244_{(a,A)}$ | **182$_{(B)}$** |
| **H5** | $281_{(ab,A)}$ | $281_{(ab,A)}$ | $201_{(b,A)}$ | $303_{(ab,A)}$ | $319_{(a,A)}$ | $272_{(ab,A)}$ | **276$_{(A)}$** |
| $\bar{x}$ | **234$_{(a)}$** | **220$_{(a)}$** | **157$_{(b)}$** | **229$_{(a)}$** | **206$_{(a)}$** | **202$_{(a)}$** | |

Significant differences were determined using Tukey's HSD test, $p < 0.05$ (capital letters: significant differences between the harvests; lower case letters: significant differences between the ecotypes); *z* = glucosinolate content

*Glucosinolate profile*

*M. oleifera* ecotypes differed in their total glucosinolate content depending on the date of harvest. In all six ecotypes, four *Moringa* glucosinolates were identified: the 4-*α*-rhamnopyranosyloxy-benzyl glucosinolate (Rhamno-Benzyl-GS) and three isomeric acetyl-4-*α*-rhamnopyranosyloxy-benzyl glucosinolates (Ac-Isomer-GSs I, II, III, Figure 2). As an exception in individual plants of ecotypes 5 and 6, only one glucosinolate, the Rhamno-Benzyl-GS, was found. Furthermore, the six *M. oleifera* ecotypes exhibited differences in their glucosinolate composition. The percentage of individual glucosinolates relative to the total glucosinolate content was determined (Figure 3, exemplary presentation based on data from the third harvest, plants from the ecotypes 5 and 6 with only Rhamno-Benzyl-GS were excluded from the calculation). Ecotypes 1 displayed a consistently higher percentage of Rhamno-Benzyl-GS and lower percentage of Ac-Isomer-GS III. A low percentage of Rhamno-Benzyl-GS and a high percentage of the three Ac-Isomer-GSs were found for ecotypes 2 and 6. Ac-Isomer-GS I was found as consistently low content levels in ecotypes 4 and 5, likewise Ac-Isomer-GS II in ecotypes 1, 4, and 5. Furthermore, the glucosinolate composition of younger and older leaves of the different *M. oleifera* ecotypes was compared, but no significant differences were observed (detailed data not shown).




1 4-α-rhamnopyranosyloxy-benzyl glucosinolate (-MS, *m/z* 570)
2-4 4 acetyl-4-α-rhamnopyranosyloxy-benzyl glucosinolate (Isomer I-III) (-MS, *m/z* 612)
5 chlorogenic acid (Isomer) (-MS, *m/z* 353)
6 neochlorogenic acid (-MS, *m/z* 353)
7 5-*p*-coumaroyl quinic acid (-MS, *m/z* 337)
8 3-*p*-coumaroyl quinic acid (-MS, *m/z* 337)
9 feruloyl quinic acid (Isomer) (-MS, *m/z* 367)
10 chlorogenic acid (-MS, *m/z* 353)
11 vitexin (-MS, *m/z* 431)
12 Isovitexin (-MS, *m/z* 431)
13 quercetin 3-*O*-glucoside (-MS, *m/z* 463)
14 kaempferol 3-*O*-glucoside) (-MS, *m/z* 447)
15 quercetin 3-*O*-(6''-malonylglucoside) (-MS, *m/z* 549)
16 kaempferol 3-*O*-(6''malonylglucoside) (-MS, *m/z* 533)
17 4-methoxycinnamic acid (internal standard) (-MS, *m/z* 177)

Fig. 2: Chromatograms of the *Moringa oleifera* glucosinolates, flavonoids, and phenolic acids

*Phenolic acid and flavonoid profile*

In *M. oleifera* leaves, the following phenolic compounds were identified: an isomer of chlorogenic acid, neochlorogenic acid, two isomers of *p*-coumaroyl quinic acid, an isomer of feruloyl quinic acid, chlorogenic acid, vitexin, isovitexin, quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside, quercetin 3-*O*-(6´´-malonylglucoside), and kaempferol 3-*O*-(6´´-malonylglucoside) (Figure 2). The main phenolic acid was identified as neochlorogenic acid and the main flavonoid was quercetin 3-*O*-(6´´-malonylglucoside). The total phenolic acid content in the leaves differed significantly between the six *M. oleifera* ecotypes (Table 2). Ecotype 5 had a significantly higher phenolic acid content than ecotype 3 (one-way ANOVA, Tukey's HSD test, $p < 0.05$). The phenolic acid content ranged between 9.86 and 17.08 µmol/g dry weights (dw), and the flavonoid content ranged between 4.47 and 10.73 µmol/g dw. Significant differences between single phenolic acids and flavonoids were identified (Table 2). Vitexin and isovitexin

were found in all plants of the *M. oleifera* ecotype 5, whereas these secondary metabolites were not present in detectable levels in the plants of ecotypes 2 and 3. No correlation was found between plant height and the phenolic acid/flavonoid content ($R^2 < 0.06$). In summary, ecotypes 4 and 5 had the highest contents of flavonoids and phenolic acids.

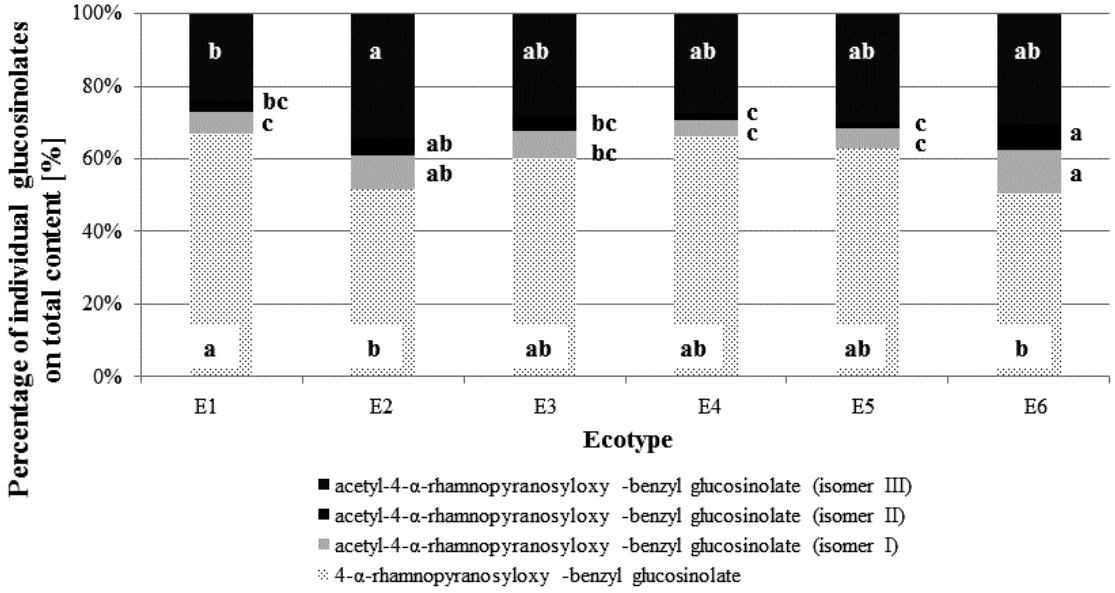



Fig. 3: Percentage of single *Moringa oleifera* glucosinolates on the total glucosinolate content in leaves at the third point of harvest (different letters significant differences between the different ecotypes within each glucosinolate, Tukey's-HSD-test, $p < 0.05$)

Tab. 2: Content of phenolic acids and flavonoids in six *Moringa oleifera* ecotypes at the third point of harvest

| | | Ecotype | | | | | |
|---|---|---|---|---|---|---|---|
| | | **E1** | **E2** | **E3** | **E4** | **E5** | **E6** |
| **Phenolic acids [µmol/g dw]** | Chlorogenic acid (Isomer) | 0.19 (a) | 0.23 (a) | 0.15 (a) | 0.22 (a) | 0.23 (a) | 0.22 (a) |
| | Neochlorogenic acid | 8.80 (a) | 10.39 (a) | 6.56 (a) | 10.45 (a) | 11.28 (a) | 11.00 (a) |
| | 5-*p*-coumaroyl quinic acid | 0.74 (ab) | 0.46 (b) | 0.57 (ab) | 1.05 (a) | 1.01 (a) | 0.81 (ab) |
| | 3-*p*-coumaroyl quinic acid | 1.77 (a) | 1.10 (a) | 1.23 (a) | 2.61 (a) | 2.32 (a) | 1.81 (a) |
| | Feruloyl quinic acid (Isomer) | 0.30 (a) | 0.16 (a) | 0.18 (a) | 0.28 (a) | 0.18 (a) | 0.14 (a) |
| | Chlorogenic acid | 1.27 (b) | 1.34 (b) | 1.17 (b) | 1.26 (b) | 2.06 (a) | 2.10 (a) |
| | **$\Sigma_{(phenolic\ acids)}$** | **11.06 (ab)** | **13.67 (ab)** | **9.86 (b)** | **15.86 (ab)** | **17.08 (a)** | **16.08 (ab)** |
| **Flavonoids [µmol/g dw]** | Vitexin | 0.12 (a) | 0.00 (a) | 0.00 (a) | 0.13 (a) | 0.10 (a) | 0.06 (a) |
| | Isovitexin | 0.22 (a) | 0.00 (b) | 0.00 (b) | 0.13 (ab) | 0.17 (ab) | 0.13 (ab) |
| | Quercetin 3-*O*-glucoside | 1.24 (a) | 0.95 (a) | 0.63 (a) | 1.89 (a) | 1.58 (a) | 1.19 (a) |
| | Kaempferol 3-*O*-glucoside) | 0.32 (a) | 0.24 (a) | 0.09 (a) | 0.11 (a) | 0.38 (a) | 0.39 (a) |
| | Quercetin 3-*O*-(6"-malonylglucoside) | 4.47 (a) | 3.61 (a) | 2.50 (a) | 6.56 (a) | 3.78 (a) | 3.03 (a) |
| | Kaempferol 3-*O*-(6"malonylglucoside) | 1.91 (a) | 1.90 (a) | 1.25 (a) | 1.90 (a) | 1.34 (a) | 1.43 (a) |
| | **$\Sigma_{(flavonoids)}$** | **8.28 (a)** | **6.70 (a)** | **4.47 (a)** | **10.73 (a)** | **7.35 (a)** | **6.23 (a)** |
| | **$\Sigma_{(phenolic\ acid\ +\ flavonoids)}$** | **19.34 (a)** | **20.37 (a)** | **14.33 (a)** | **26.59 (a)** | **24.43 (a)** | **22.31 (a)** |

Significant differences within each phenolic acid or flavonoid between the ecotypes, Tukey's-HSD-test, $p < 0.05$

**Influence of water deficiency and sulfur fertilization on biomass, height, and secondary metabolite content of *M. oleifera* ecotypes**

The influence of water deficiency and sulfur fertilization on biomass accumulation, plant height, and secondary metabolite profile (glucosinolate, phenolic acids, and flavonoids) was analyzed in six *M. oleifera* ecotypes. Contrary to the results of the second point of harvest (plants were exposed to drought stress, Suppl. Figure 2), plants showed stronger reactions at the beginning of a repeated water limitation (third point of harvest, Figure 4). Therefore, the results of the third point of harvest were presented in more detail in the present work. At the third point of harvest, the plants in the normal cultivation variant were significantly taller and had a higher biomass than plants grown under water limitation (Figure 4, Tukey's HSD test, $p < 0.05$). The glucosinolate content was overall significantly higher in the water deficiency variant in comparison to the normal variant (Tukey's HSD test, $p < 0.05$). Cultivation conditions had different impacts on biomass accumulation, plant height, and glucosinolate content of the six ecotypes (Figure 4). No statistically significant correlation between the cultivation variant and the ecotype was found for the glucosinolate content, biomass, and plant height (two-way ANOVA, $p < 0.05$). Although no influence of the cultivation variant on the flavonoid content was identified for the single ecotypes, statistical differences in the phenolic acid content were found (Figure 5, ecotype 1 and 4). With regard to the sum of phenolic acids and flavonoids, ecotype 1 and 2 had significantly higher contents in the sulfur variant in comparison to the normal variant. Ecotype 3 and 4 had significant higher contents in the drought variant in comparison to the normal variant (Tukey's HSD test, $p < 0.05$). A statistically significant interaction between the cultivation variant and ecotype was identified (two-way ANOVA, $p < 0.01$). In general, the effect of the cultivation variant on the plant biomass and content of glucosinolates, phenolic acids, and flavonoids strongly depended on the ecotype.

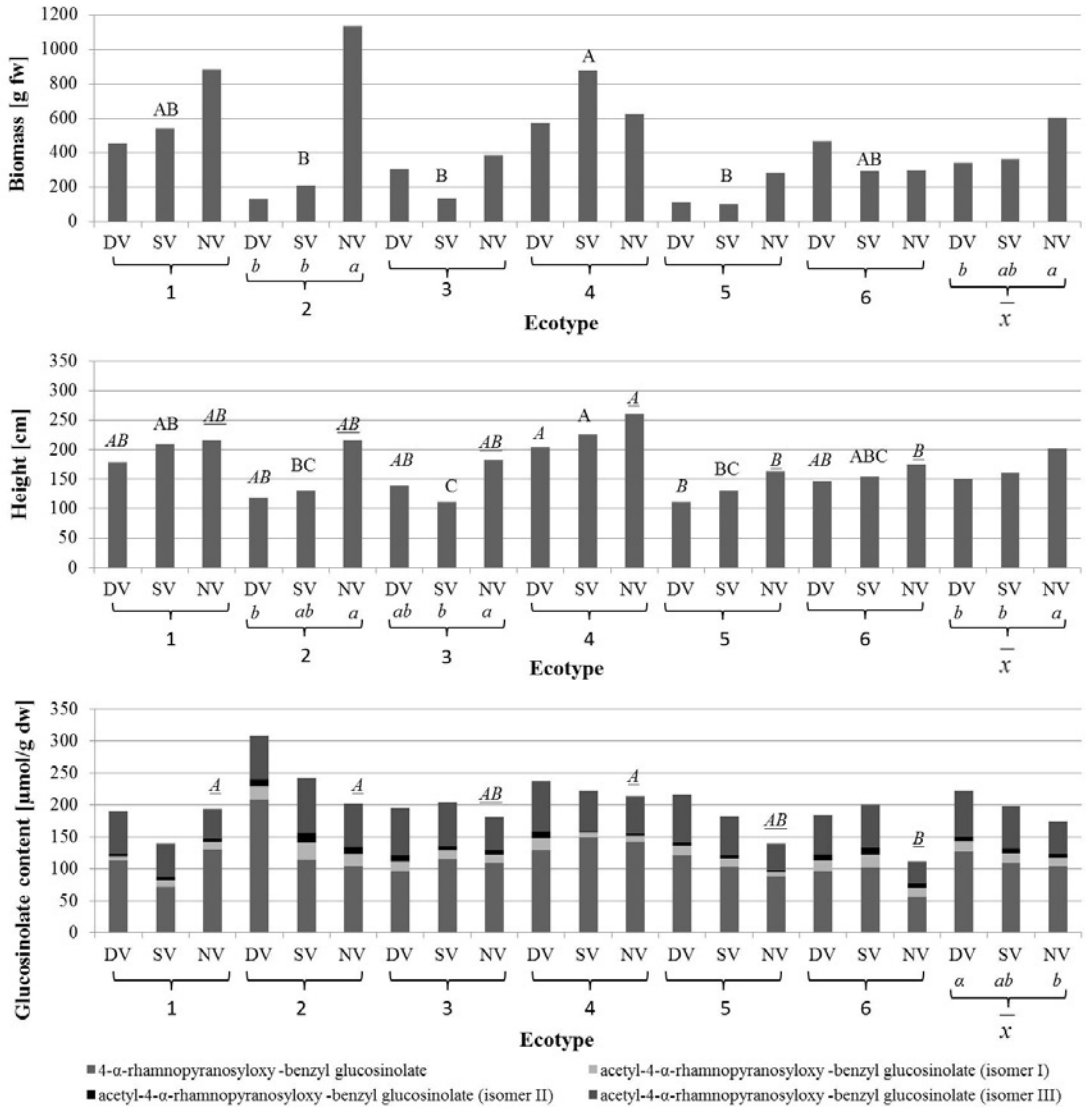



Fig. 4: Influence of cultivation variants on biomass, height, and glucosinolate content of six *Moringa oleifera* ecotypes at the third point of harvest (lower case letters = significant differences between the cultivation variants within each ecotype; capital letters = significant differences between the different ecotypes within one cultivation variant; Tukey's HSD test, $p < 0.05$; DV = water deficiency variant; SV = sulfur variant; NV = normal variant)

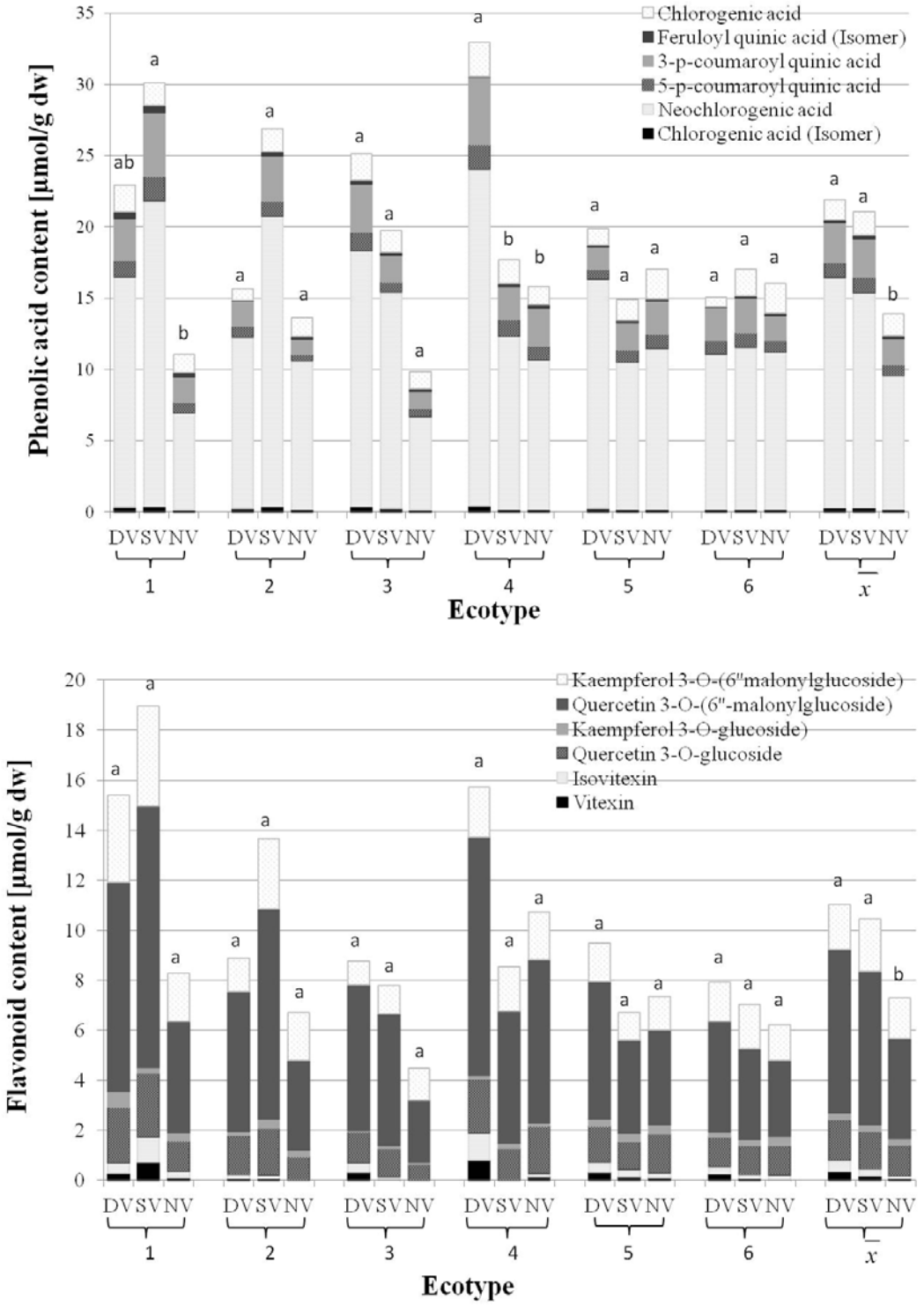



Fig. 5: Phenolic acid and flavonoid content of six *Moringa oleifera* ecotypes in different cultivation variants at the third point of harvest (lower case letters = significant differences in total contents between the cultivation variants within each ecotype; Tukey's HSD test, $p < 0.05$; DV = water deficiency variant; SV = sulfur variant; NV = normal variant)

## Discussion

*M. oleifera* ecotypes are characterized by phenotypic differences, such as the shape of leaves, the production of leaf biomass, the plant height, and the secondary metabolite profile, as reported in other studies (Bennett et al., 2003; Doerr et al., 2009; Palada & Chang). Therefore, the intensive cultivation of *M. oleifera* ecotypes should be associated with a high yield of leaf biomass and secondary metabolites. Differences in the secondary metabolite content and composition have not been the basis for selecting ecotypes for intensive cultivation until today, although health-promoting effects of chemical compounds of *M. oleifera* are commonly described next to its nutritional value (Fahey, 2005; Ferreira et al., 2008). The six different *M. oleifera* ecotypes used in this study showed morphological (height and biomass) and chemical (glucosinolates, phenolic acids, and flavonoids) phenotypic differences under similar cultivation conditions. Ecotypes 1 and 4 were identified as having the highest yields of biomass and glucosinolate content (Table 1), and ecotypes 4 and 5 were identified as having the highest yields of phenolic acids and flavonoids, followed by ecotypes 1 and 6 (Table 2). It is most likely that the diverse characteristics in the phenotype (growth characteristics and chemical profile) found in our analysis are fixed genetically because the ecotypes arise from germplasm collection and were cultivated and analyzed in a similar manner. To exclude the effects of the environment on the analyzed parameter, seeds were sown and planted at the same time, so that each plant was of the same age and at the same developmental stage. Interestingly, most trees of ecotypes 5 and 6 in the present study contained only one glucosinolate, the Rhamno-Benzyl-GS, but none of the three Ac-Isomer GSs. This observation was not described by other authors. Shockey et al. (2003) reported that acyl-activating enzymes are present in all living organisms. According to these authors, *Arabidopsis thaliana* L. possesses an acyl-activating enzyme superfamily containing 63 different genes. It is conceivable that different enzyme activity in the plants could lead to a lack of acetylated glucosinolates in some plants of the *M. oleifera* ecotypes.

As described by Brown et al. (2003) for *A. thaliana*, we found higher glucosinolate contents in the younger leaves of *M. oleifera* than in the older leaves, whereas no differences in glucosinolate composition were determined (Suppl. Table 2). This could

be explained by glucosinolate concentration dropping and stabilizing during leaf expansion (Brown et al., 2003).

Consistent with findings of several authors in different glucosinolate containing plant species (Doerr et al., 2009; Farnham et al., 2004), the glucosinolate content of the *M. oleifera* plants tended to increase as the plant matured. Although the glucosinolate content increased for all ecotypes at approximately 50 – 100%, the biomass accumulation increased at different degrees depending on the ecotype. Analysis using different *Salix* clones, a tree species that is very similar in its growth characteristics and high content of secondary metabolites (salicylates) to *M. oleifera*, showed that the amount of shoots and the biomass increased in trees of three different species from the first to the second year after planting (Förster, 2010). On the one hand, this increase can be attributed to different environmental conditions at the harvests (temperature, day length, and solar radiation). On the other hand, the plant became better adapted over time (root penetration in the rhizosphere and access to existing water and nutrition sources). The ecotypes seem to be able to adapt to the set environmental conditions in a different speed and degree because to a variable increase in biomass accumulation over the five harvest points. A possible influence of plant age on phenolic acids and flavonoids will not be discussed at this point because these secondary metabolites were only determined at the second and third point of harvest.

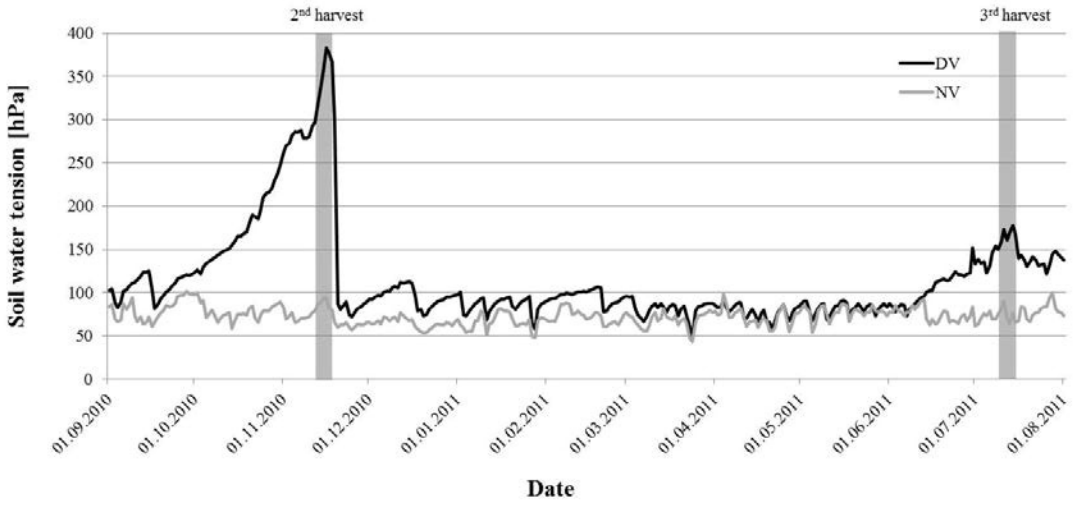
Different authors have described the influence of nitrogen and sulfur fertilization (Ahmad et al., 2007; Aires et al., 2006; Omirou et al., 2009) and different water regimes (Khan et al., 2010) on the glucosinolate content and growth of plants. Our analysis did not show a consistent result of either sulfur fertilization or water deficiency on growth and secondary metabolite accumulation in *M. oleifera*. Under water limitation (second point of harvest) ecotypes showed no differences in biomass accumulation and glucosinolate content (Suppl. Figure 2). With repeated moderate water limitation (third point of harvest, soil water tension did not exceed the range of the field capacity, Suppl. Table 3) biomass and plant height was reduced; on average across all ecotypes, the glucosinolate content increased in comparison to the normal variant (Figure 4). The content of phenolic acids (more than flavonoids) at the third point of harvest was significant lower in the normal cultivation variant in comparison to the sulfur variant and under water limitation (Figure

5). Growth and glucosinolate content were not significantly influenced by sulfur fertilization. Additional sulfur input could not be identified in soil analyses two month after fertilization, indicating a complete sulfur uptake of plants. For further studies, more frequent and higher sulfur fertilization is recommended. Clearly, all effects are strongly ecotype-dependent. To our knowledge, there are no studies available about the influence of sulfur and water deficiency on the growth and secondary metabolite content of *M. oleifera*. *M. oleifera* seems to be an intermediate between annual brassicaceous plants that contain glucosinolate and fast-growing tree species; this makes a comparison of the growth as well as secondary metabolite development of *M. oleifera* with other plants described in the literature very difficult. Furthermore, this rapidly growing tree is cut back several times a year (Fuglie & Sreeja (2001), which makes it even more difficult to compare growth parameters with other trees. Regular pruning, as also performed with the trees in the present study, promotes a compact and dense habitus, and the trees were much stronger branched, resulting in an increased biomass accumulation. Clearly, the results of the present study showed that different cultivation variants influenced the morphological and chemical phenotype of single *M. oleifera* ecotypes differently.

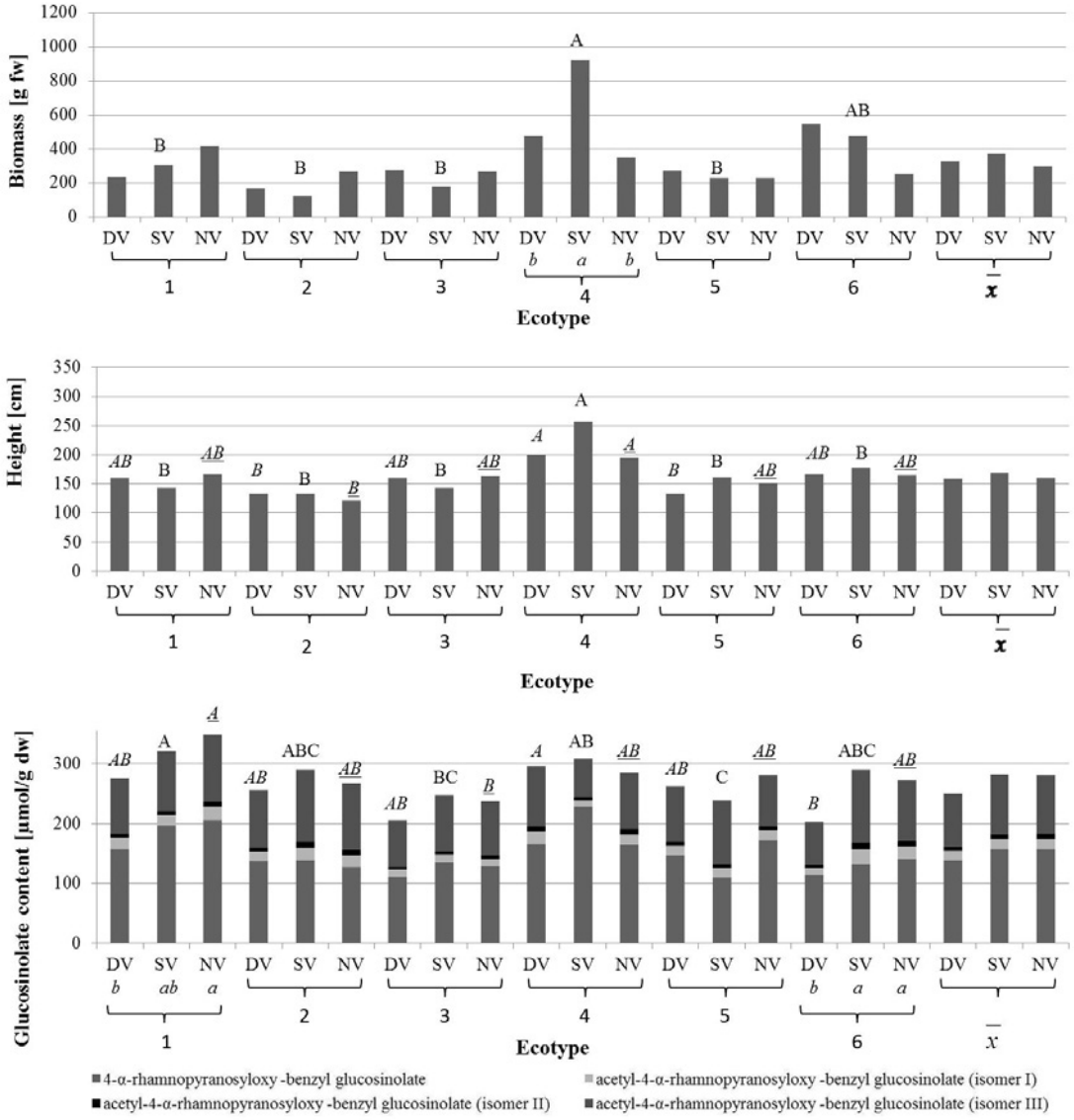
A trade-off between biomass accumulation and secondary metabolite production, for example, as observed in the fast-growing tree species *Salix sericea* Marsh by Nichols-Orians et al. (1993), was not generally the case for the *M. oleifera* ecotypes investigated here. Ecotypes 1, 2, and 4 each had a biomass twice as high as those of ecotypes 5 and 6. At the same time, the glucosinolate content of ecotypes 1, 2, and 4 - 6 was not significantly different (Table 1). These findings are not in agreement with the trade-off between growth and defense over a resource-availability gradient, as stated in the growth-differentiation-balance hypothesis. However, on average across all ecotypes, the biomass accumulation was significantly higher in the normal cultivation variant in comparison to plants grown under drought stress at the third point of harvest. We found the opposite pattern for the glucosinolate content. These findings confirm results from Zhang et al. (2008) or Schreiner et al. (2009) that determined increased glucosinolate contents under moderate drought stress in *Brassica carinata* A. Braun and *Brassica rapa* L. ssp. *rapa*, whereas other authors found contradictory results (e. g. Gutbrodt et al., 2011). The content of phenolic acids and flavonoids was not dependent on the biomass and plant height of *M. oleifera*.

Observed phenotypic differences under the same environmental conditions as in the present study can be attributed to plant genetics. The effects of sulfur and water deficiency (different cultivation variants) on six *M. oleifera* ecotypes, as a tree containing glucosinolate, had not previously been the subject of investigation. The phenotype of *M. oleifera* plants depended on the analyzed environmental factors (water deficiency and sulfur supply). This demonstrates that plant genetics determine the biomass production, growth, and content of phytochemicals, although environmental factors can modulate these parameters (Doerr et al., 2009). The presented results showed that no general cultivation recommendations can be made. The optimal cultivation of *M. oleifera* seems to be highly dependent on the ecotype. Nevertheless, potential suitable ecotypes, meeting the criteria of an optimal leaf yield (high biomass) with high levels of nutritional and health-promoting properties (glucosinolate, phenolic acid, and flavonoid content) in addition to a capability to withstand water limitation could be identified within the present study: Ecotypes 1 (TOT4880, origin USA) and 4 (TOT7267, origin India). Because of an enormous suggestibility of growth and secondary metabolites by the environment, findings should be proved in field trials in *M. oleifera* cultivation areas.

## Supplemented Figures and Tables




Suppl. Fig. 1: Soil water tension in the bed of the *Moringa oleifera* ecotypes of normal cultivation and the water deficiency variant from September 2010 till August 2011




Suppl. Fig. 2: Influence of cultivation variants on biomass, height, and glucosinolate content of six *Moringa oleifera* ecotypes at the second point of harvest (lower case letters = significant differences between the cultivation variants within each ecotype; capital letters = significant differences between the different ecotypes within one cultivation variant, Tukey's HSD test, $p < 0.05$, DV = water deficiency variant, SV = sulfur variant, NV = normal variant)

Suppl. Tab. 1: Growing parameters in the three different growing variants of *Moringa oleifera*

| | **Growing variant** | | |
|---|---|---|---|
| **Parameter** | **Normal variant** | **Sulfur variant** | **Water deficiency variant** |
| **Irrigation** | Continuous drip irrigation | Continuous drip irrigation | Interruption of drip irrigation from August – November 2010 and June – September 2011 |
| **Soil water tension** | 50 – 100 hPa | 50 – 100 hPa | 380 hPa, 175 hPa |
| **Nebulization** | Interruption of nebulization from June 2010 till August 2011 | Interruption of nebulization from June 2010 till August 2011 | Interruption of nebulization from June 2010 till August 2011 |
| **Fertilization (annual)** | 7.04 g/m$^2$ nitrogen (two times), 2.56 g/m$^2$ phosphorus, 5.76 g/m$^2$ potassium | 7.04 g/m$^2$ nitrogen (two times), 2.56 g/m$^2$ phosphorus, 5.76 g/m$^2$ potassium 0.704 g/m$^2$ sulfur | 7.04 g/m$^2$ nitrogen (two times), 2.56 g/m$^2$ phosphorus, 5.76 g/m$^2$ potassium |

Suppl. Tab. 2: Calculated glucosinolate profit of harvested leaf material of six ecotypes of *Moringa oleifera* (first point of harvest)

| | | Ecotype | | | | | |
|---|---|---|---|---|---|---|---|
| | | E1 | E2 | E3 | E4 | E5 | E6 |
| GS[z] content in different aged leaves [µmol/g dw[y]] | young | 148 | 155 | 101 | 144 | 129 | 110 |
| | middle | 110 | 91 | 78 | 72 | 88 | 78 |
| | old | 88 | 76 | 72 | 41 | 62 | 63 |
| | $\bar{x}$ | 118 | 107 | 84 | 86 | 93 | 84 |
| Leaf water content [%] | young | 86 | 85 | 83 | 85 | 85 | 82 |
| | middle | 80 | 80 | 82 | 82 | 81 | 81 |
| | old | 80 | 79 | 80 | 81 | 80 | 81 |
| | $\bar{x}$ | 82 | 81 | 82 | 82 | 82 | 81 |
| Leaf GS content [µmol/g fw[x]] | $\bar{x}$ | 21 | 20 | 15 | 15 | 17 | 16 |
| Plant biomass [g fw[x]] | | 104 | 253 | 122 | 331 | 154 | 94 |
| Proportion of leaves [%] | | 58 | 54 | 64 | 68 | 63 | 61 |
| Leaf biomass [g fw[x]] | | 60 | 137 | 78 | 225 | 97 | 57 |
| GS content of harvested leaf material [µmol/ leaf material of one plant] | | 1281 | 2777 | 1181 | 3484 | 1624 | 915 |

*z* = glucosinolate, *y* = dry weight , *x* = fresh weight

Suppl. Tab. 3: Temperature, humidity, and water content of the air in the greenhouse at the second and third point of harvest

| | **Second point of harvest** | **Third point of harvest** |
|---|---|---|
| **Lowest temperature [°C]** | 14.0 | 15.0 |
| **Corresponding humidity [%]** | 78.2 | 95.0 |
| **Water content of the air* [g/kg]** | 7.7 | 9.9 |
| **Hightest temperature [°C]** | 16.9 | 26.2 |
| **Corresponding humidity [%]** | 75.7 | 57.6 |
| **Water content of the air* [g/kg]** | 8.9 | 12.0 |

* calculated by the Mollier-h-x-diagram

## References

Abreu, I. N. de & Mazzafera, P. (2005): Effect of water and temperature stress on the content of active constituents of *Hypericum brasiliense* Choisy. *Plant Physiology and Biochemistry* 43: 241-248.

Ahmad, G.; Jan, A.; Arif, M.; Jan, M. T. & Khattak, R. A. (2007): Influence of nitrogen and sulfur fertilization on quality of canola (*Brassica napus* L.) under rainfed conditions. *Journal of Zhejiang University SCIENCE B* 8: 731-737.

Aires, A.; Rosa, E. & Carvalho, R. (2006): Effect of nitrogen and sulfur fertilization on glucosinolates in the leaves and roots of broccoli sprouts (*Brassica oleracea* var. *italica*). *Journal of the Science of Food and Agriculture* 86: 1512-1516.

Bellostas, N.; Sørensen, J. C.; Nikiema, A.; Sørensen, H.; Pasternak, D. & Kumar, S. (2010): Glucosinolates in leaves of *Moringa* species grown and disseminated in Niger. *African Journal of Agricultural Research* 5: 1336-1340.

Bennett, R. N.; Mellon, F. A.; Foidl, N.; Pratt, J. H.; Dupont, M. S.; Perkins, L. & Kroon, P. A. (2003): Profiling glucosinolates and phenolics in vegetative and reproductive

tissues of the multi-purpose trees *Moringa oleifera* L. (Horseradish Tree) and *Moringa stenopetala* L. *Journal of Agricultural and Food Chemistry* 51: 3546-3553.

Bloem, E.; Haneklaus & S.; Schnug, E. (2007): Comparative effects of sulfur and nitrogen fertilization and post-harvest processing parameters on the glucotropaeolin content of *Tropaeolum majus* L. *Journal of the Science of Food and Agriculture* 87: 1576-1585.

Brown, P. D.; Tokuhisa, J. G.; Reichelt, M. & Gershenzon, J. (2003): Variation of glucosinolates accumulation among different organs and developmental stages of *Arabidopsis thaliana*. *Phytochemistry* 62: 471-481.

Dai, J. & Mumper, R. J. (2010): Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules* 15: 7313-7352.

Dam, N. M. van; Tytgat, T. O. G. & Kirkegaard, J. A. (2009): Root and shoot glucosinolates: A comparison of their diversity, function and interactions in natural and managed ecosystems. *Phytochemistry Reviews* 8: 151-186.

Doerr, B.; Wade, K. L.; Stephenson, K. K.; Reed, S. B. & Fahey, J. W. (2009): Cultivar effect on *Moringa oleifera* glucosinolate content and taste: A pilot study. *Ecology of Food and Nutrition* 48: 100-211.

Fahey, J. W. (2005): *Moringa oleifera*: A Review of the medical evidence for its nutritional, therapeutic, and prophylactic properties. Part 1. *Trees for Life Journal* (http://www.tfljournal.org/article.php/20051201124931586), (Zugriff 8.5.2017).

Farnham, M. W.; Wilson, P. E.; Stephenson, K. K. & Fahey, J. W. (2004): Genetic and environmental effects on glucosinolate content and chemoprotective potency of broccoli. *Plant Breeding* 123: 60-65.

Ferreira, P. M. P.; Farias, D. F.; Oliveira, J. T. d. A. & Carvalho, A. d. F. U. (2008): *Moringa oleifera*: bioactive compounds and nutritional potential. *Revista Nutrição* 21: 431-437.

Förster, N. (2010): Eignung unterschiedlicher salicylathaltiger *Salix*-Klone für die Arzneimittelindustrie. Berliner ökophysiologische und phytomedizinische Schriften, Band 7, Der Andere Verlag, Tönning, Lübeck und Marburg, 103-105.

Förster, N.; Ulrichs, C.; Schreiner, M.; Müller, C. T. & Mewis, I. (2015): Development of a reliable extraction and quantification method for glucosinolates in *Moringa oleifera*. *Food Chemistry* 166: 456-64.

Fuglie L. J. & Sreeja K. V. (2001): Cultivation of *Moringa*. In: Miracle Tree: The multiple attributes of *Moringa*. Ed. Fuglie I. J., CTA, CWS, Dakar, Senegal, 153-158.

Gamatie, M. (2001): *Moringa oleifera* management systems in the river Niger valley: The case of Sarando area, Konferenz: Development potential of *Moringa* products? (29. Oktober – 2. November 2001, Dar es Salaam, Tanzania).

Gutbrodt, B.; Mody, K. & Dorn, S. (2011): Drought changes plant chemistry and causes contrasting responses in lepidopteran herbivores. *Oikos* 120: 1732-1740.

Halkier, B. A. & Gershenzon, J. (2006): Biology and biochemistry of glucosinolates. *Annual Review of Plant Biology* 57: 303-333.

Hernández, I.; Alegre, L. & Munné-Bosch, S. (2004): Drought-induced changes in flavonoids and other low molecular weight antioxidants in *Cistus clusii* grown under Mediterranean field conditions. *Tree Physiology* 24: 1303-1311.

Khan, M. A. M.; Ulrichs, C. & Mewis, I. (2010): Influence of water stress on the glucosinolate profile of *Brassica oleracea* var. *italica* and the performance of *Brevicoryne brassicae* and *Myzus persicae*. *Entomologia Experimentalis et Applicata* 137: 1-8.

Li, J.; Zhu, Z. & Gerendás, J. (2008): Effects of nitrogen and sulfur on total phenolics and antioxidant activity in two genotypes of leaf mustard. *Journal of Plant Nutrition* 31: 1542-1655.

Mewis, I.; Smetanska, I. M.; Müller, C. & Ulrichs, C. (2011): Specific poly phenolic compounds in cell culture of *Vitis vinifera* L. cv. Gamay Fréaux. *Applied Biochemistry and Biotechnology* 164: 148-161.

Mithen, R.; Bennett, R. & Marquez, J. (2010): Glucosinolate biochemical diversity and innovation in the Brassicales. *Phytochemistry* 71: 2074-2086.

Nichols-Orians C. M.; Fritz R. S. & Clausen T. P. (1993): The genetic basis for variation in the concentration of phenolic glycosides in *Salix sericea*: Clonal variation and sex-based differences. *Biochemical Systemetics and Ecology* 21: 535-542.

Olivier, C.: Intensive *Moringa oleifera* cultivation in the north of Senegal. (http://www.moringanews.org/documents/Leafproduction.doc), (Zugriff: 8.5.2017).

Omirou, M. D.; Papadopoulou, K. K.; Papastylianou, I.; Constantinou, M.; Karpouzas, D. G.; Asimakopoulos, I. & Ehaliotis, C. (2009): Impact of nitrogen and sulfur fertilization on the composition of glucosinolates in relation to sulfur assimilation in different plant organs in broccoli. *Journal of Agricultural and Food Chemistry* 57: 9406-9417.

Palada, M. C. & Chang, L. C. (2003): Suggested cultural practices for *Moringa*. International cooperators´ guide, AVRDC pub # 03-545.

Palada, M. C. (1995): *Moringa* (*Moringa oleifera* Lam.): A versatile tree crop with horticultural potential in the subtropical United States. *Hortscience* 31: 794-797.

Pascale, S. de; Maggio, A.; Pernice, R.; Fogliano, V. & Barbieri, G. (2007): Sulphur fertilization may improve the nutritional value of *Brassica rapa* L. subsp. *sylvestris*. *European Journal of Agronomy* 26: 418-424.

Radovich, T.: Farm and forestry, production and marketing profile for *Moringa* (*Moringa oleifera*). (http://www.agroforestry.net/scps/Moringa_specialty_crop.pdf), (Zugriff: 8.5.2017).

Saha, P.; Jena, R. C.; Sahoo, B.; Sahoo, K.; Lenka, A. & Adansonia, A. (2012): Sufficing neutraceutical rich multipurpose leafy vegetable on earth: *Moringa*. *Odisha Review*, Oktober, 72-80.

Schreiner, M.; Beyene, B.; Krumbein, A. & Stützel, H. (2009): Ontogenetic changes of 2-propenyl and 3-indolylmethyl glucosinolates in *Brassica carinata* leaves as affected by water supply. *Journal of Agricultural and Food Chemistry* 57: 7259-7263.

Shockey, J. M.; Fulda, M. S. & Browse, J. (2003): *Arabidopsis* contains a large superfamily of acyl-activating enzymes. Phylogenetic and biochemical analysis reveals a new class of acyl-coenzyme a synthetases. *Plant Physiology* 132: 1065-1076.

Siddhuraju, P. & Becker, K. (2003): Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringa oleifera* Lam.) leaves. *Journal of Agricultural and Food Chemistry* 51: 2144-2155.

Sousa, C; Pereira, D. M.; Pereira, J. A.; Bento, A.; Rodrigues, M. A.; Dopico-Garcia, S., Valentão, P.; Lopes, G.; Ferreres, F.; Seabra, R. M. & Andrade, P. B. (2008): Multivariate analysis of Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) phenolics: Influence of fertilizers. *Journal of Agricultural and Food Chemistry* 56: 2231-2239.

Thurber, M. D. & Fahey, J. W. (2009): Adoption of *Moringa oleifera* to combat under-nutrition viewed through the lens of the "Diffusion of Innovations" theory. *Ecology of Food and Nutrition* 48: 212-225.

Vallejo, F.; Tomás-Barberán, F. A. & García-Viguera, C. (2003): Effects of climatic and sulphur fertilizsation conditions, on phenolic compounds and vitamin C, in the inflorescences of eight broccoli cultivars. *European Food Research and Technology* 216: 395-401.

Zhang, H.; Schonhof, I.; Krumbein, A.; Gutezeit, B.; Li, L.; Stüztel, H. & Schreiner, M. (2008): Water supply and growing season influence glucosinolate concentration and composition in turnip root (*Brassica rapa* ssp. *rapifera* L.). *Journal of Plant Nutrition and Soil Science* 171: 255-265.

## Kapitel II - *In vitro*-Kultur von *M. oleifera*

Da *M. oleifera* zwar als trocken- jedoch nicht frostresistent gilt, ist eine ganzjährige Kultivierung in der gemäßigten Klimazone der Erde nicht möglich. Optimales Wachstum erreicht der Baum bei Temperaturen zwischen 25 – 35 °C und bei jährlichen Niederschlagsmengen zwischen 250 – 600 mm (Palada & Chang, 2003). Um ganzjährig Blattmaterial für die geplanten Versuche zur Verfügung zu haben, sollte *M. oleifera* in die *in vitro*-Kultur überführt werden. Zum einen würde die Möglichkeit geschaffen werden die Pflanzen außerhalb der Vegetationsperiode zu vermehren. Eine anschließende schnelle Freiland-Etablierung eines großen Pflanzenbestandes zur Gewinnung von ausreichend Blattmaterial innerhalb der Vegetationsperiode in den gemäßigten Breiten wäre denkbar. Weiterhin wäre auch eine Gewinnung von sekundären Inhaltsstoffen direkt aus der *in vitro*-Kultur denkbar. Pflanzliche Zellkulturen wurden bereits von vielen Autoren verwendet, um sekundäre Inhaltstoffe für die Medizin oder Nahrungsindustrie zu generieren. So konnten z. B. aus einer Suspensionskultur von *Vitis vinifera* Anthocyane gewonnen werden und aus einer Kalluskultur von *Tropaeolum majus* das Benzylglucosinolat (Decendit & Mérillon 1996; Meyer & Van Staden 1995; Wielanek & Urbanek 1999). Folgende Fragestellungen wurden daher untersucht:

- *Besteht die Möglichkeit M. oleifera in vitro zu etablieren und zu vermehren, um diese in der Vegetationsperiode auszupflanzen (ex vitro) und zur Gewinnung von Blattmaterial zu nutzen?*
- *Bei welchen in vitro-Wachstumsbedingungen können die höchsten Vermehrungsraten erzielt werden?*
- *Ist es möglich sekundäre Inhaltsstoffe (Glucosinolate) in in vitro-Pflanzen von M. oleifera anzureichern, um diese für eine Inhaltsstoffgewinnung zu verwenden?*

Die in diesem Kapitel dargestellten Fragenstellungen werden in folgender Publikation behandelt:

> Förster, N.; Mewis, I. & Ulrichs, C. (2013): *Moringa oleifera* – Establishment and multiplication of different ecotypes *in vitro*. *Gesunde Pflanzen* 65: 21-31.

## Abstract

To obtain healthy plant material from *Moringa oleifera* regardless of season, weather, and degree of infestation, a procedure to establish an *in vitro* culture of *M. oleifera* from nodes using 0.2% mercury chloride was developed. It was not possible to create an *in vitro* culture of *M. oleifera* from seeds. Nodes were cultivated on MS medium with different concentrations of benzylaminopurine (BAP) and agar contents to find the best conditions for rapid growth and optimum multiplication. The highest multiplication ability of the different plant parts, especially the base parts of *M. oleifera in vitro* plants, was observed after three weeks of cultivation on MS medium with 0.5 mg/l BAP. Callus formation increases with increased BAP concentration (0, 0.5, 0.75, 1 mg/l BAP). Furthermore, the use of two phytohormones - indole-3-acetic acid and thidiazuron – led to very strong callus formation of adaxial and abaxial orientated leaves on MS medium. This formation was only observed for material that was light induced for 24 hours prior to cultivation under dark conditions. Analysis of the glucosinolate content of *M. oleifera* leaves revealed a different glucosinolate profile of plants cultivated *in vitro* and in soil beds in the greenhouse. Whereas in greenhouse leaves rhamnopyranosyloxy-benzyl glucosinolates were abundant, the precursor benzyl glucosinolate was found in *in vitro* cultures.

## Introduction

*Moringa oleifera* is the most commonly cultivated and widespread of the 12 species in the genus *Moringa* (Olson, 2002). Originally native to the sub-Himalayan tracts of India, Pakistan, Bangladesh, and Afghanistan (Ramachandran et al., 1980; Olson, 2002), today *M. oleifera* has spread to many tropical locations such as West, East and South Africa, tropical Asia, Latin America, the Caribbean, Florida, and the Pacific Islands (Fahey, 2005). All parts of *Moringa* are used by humans in different ways, such as for medicine (leaves, roots, barks, fruits, flowers, seed oil), functional food (leaves, flowers), nutraceuticals (leaves, fruits, flowers, immature pods), and water purification (seeds) (Anwar et al., 2007). Usage of the leaves is of particular significance because they contain high concentrations of health-relevant, nutritious ingredients. The leaves of *Moringa* are an important source of highly digestible proteins (0.057 g/g fresh weight (fw)), calcium

(6.38 mg/g fw), iron (0.092 mg/g fw), vitamin C (ascorbate: 2.49 mg/g fw), and antioxidant compounds such as flavonoids (flavonol content: 6.2 mg/g dry weight (dw)), phenolics (74-210 µmol/g dw), carotenoids (β-carotene: 1.1 - 2.8 µmol/g dw), and vitamin E (α-tocopherol: 0.7 - 1.1 µmol/g dw) (Sultana & Anwar, 2008; Yang et al., 2006). For this reason, *Moringa* acts as an important food source to fight against "hidden hunger". Furthermore, *Moringa* does not lose its leaves in the dry season and is very dry resistant. In addition to being used to combat malnutrition, *Moringa* is especially used by South African tribes to treat inflammations, infectious diseases, cardiovascular problems, gastrointestinal illnesses, and nervous disorders (Cáceres et al., 1992; Eilert et al., 1981; Faizi et al., 1994; Gilani et al., 1994; Nadkarni, 1976; Nikkon et al., 2003). Extracts, decoctions, pulps, oils, salves, and powders are made to treat or prevent diseases and infections. Wide knowledge of the healing powers of *Moringa* is based on the know-how of tribes and oral tradition. Scientific verification to test the medical activity of the plant is very fragmentary (Sampson, 2005; Talalay & Talalay, 2001). Different medical studies show antibiotic, diuretic, antispasmodic, anti-inflammatory, hypotensive, antioxidative, or anticancerogenic effects of the *Moringa* tree (Cáceres et al., 1992; Eilert et al., 1981; Gilani et al., 1994; Guevara et al., 1999; Siddhuraju & Becker, 2003). What is characteristic to *Moringa* is that all plant parts contain glucosinolates (Bennett et al., 2003). These secondary metabolites are believed to play a major role in the tree's healing effects (Eilert et al., 1981; Makonnen et al., 1997). Comparable to other species containing glucosinolates, *M. oleifera* has a very high concentration of these secondary metabolites (Förster et al., 2011; Yang et al., 2006).

Due to its origin in the subtropics and tropics, *M. oleifera* is not frost resistant. It is therefore impossible to grow this perennial tree in open land cultivation in temperate zones. Greenhouse cultivation is the only option, although the tree loses its leaves in winter (data not shown). For this reason, leaf material cannot be produced for experiments until new shoots appear again in spring. In addition, *M. oleifera* is highly susceptible to infestation with aphids, thrips, and white flies in the growing season in the temperate zone. These insect attacks cause great damage to the foliar surface, leading to a decline in total biomass. The establishment of an *in vitro* culture of *M. oleifera* will lead to production of healthy plant material free of bacteria, fungi, other animal pests, and viruses all-year. Rapid propagation of the plant can be achieved using specific media and

phytohormones. Moreover, it takes much less time for a plant to develop from a seed or node into an intact plant than in *ex situ* cultivation. It would be possible to conduct a larger number of experiments in a shorter period of time and under controlled conditions.

Furthermore, *in vitro* cultures can be used to fabricate secondary metabolites. Naturally, many plants produce special secondary metabolites to protect themselves against herbivores and pathogens or as a signaling compound. Moreover, these ingredients often have positive and negative effects on human health. Frankel et al. (1993) reported that phenolic compounds in red wine, such as anthocyanins, have potent antioxidant properties. Plant cell cultures are therefore often used in medicine or the food industry to produce these secondary metabolites (Decendit & Mérillon, 1996). Various authors show that it is possible to gain anthocyanins by cultivating cells of *Vitis vinifera* in a suspension culture (Decendit & Mérillon, 1996; Meyer & van Staden, 1995). Wielanek & Urbanek (1999) observed the production of benzyl glucosinolate in callus cultures of *Tropaeolum majus*.

The aim of the study was to obtain a sterile initial population of *in vitro* plants. The influence of different phytohormones and agar concentrations on growth and multiplication parameters was examined. These experiments are the basis for establishing callus cell cultures *in vitro* and for comparing glucosinolate accumulation in cell cultures with *M. oleifera* plants *in vitro* in further investigations.

## Materials and methods

### Media used

MS medium developed by Murashige & Skoog (1962) was used as the basis for establishing and growing nodes. The pH was set at 5.8 prior to sterilization. The MS medium was altered in agar and phytohormone concentration and composition (benzylaminopurine (BAP), indole-3-acetic acid (IAA), thidiazuron (TDZ)). The MS media with BAP or IAA were autoclaved for sterilization; the TDZ solution was filter sterilized. IAA was used as a natural auxin. TDZ is known to induce a variety of cytokinin responses.

**Culture conditions**

In all experiments, the plants were cultivated at 21 °C on shelves in a growth chamber (walk-in-chamber) with a 16-hour photoperiod. *In vitro* plants were lighted (1,000 lux).

**Establishment of an *in vitro* culture**

An *in vitro* culture of *M. oleifera* was established to yield healthy leaf material for different experiments the whole year round. Particularly, the aim of the work was to establish a shoot *in vitro* culture.

*Disinfection experiments with sodium hypochlorite and mercury chloride on seeds*

Seeds of two Thai ecotypes of *M. oleifera* were initially incubated by stirring in ethanol (70%) for 1 min. Following under the clean bench, the seeds were washed with sodium hypochlorite (2, 5, 10% NaOCl) and mercury chloride (0.2% $HgCl_2$) with one drop of Tween in 50 ml liquid (reduction of the surface tension). Seeds were incubated for 5, 15, 30, and 45 min in the sodium hypochlorite solution and for 3, 5, and 7 min in mercury chloride (five replications). The seeds were washed three times with sterile water and transferred to sample tubes with 20 ml MS medium with 8 g/l agar.

*Disinfection experiments with mercury chloride on nodes*

Seeds were sowed in soil filled pots in the greenhouse to establish a shoot *in vitro* culture of *M. oleifera*. Two months after sowing, nodes were cut from the shoot tips. The plant material was used for a disinfection test.

In the first experiment, nodes from two Thai ecotypes of *M. oleifera* (ecotypes originate from the gene bank of the Asian Vegetable Research and Development Center (AVRDC) in Taiwan) were disinfected. The nodes harvested remained in tap water until disinfection to prevent dehydration. Per experimental repetition (the experiment was repeated three times), nodes were initially incubated and agitated in ethanol (70%) for 1 min. The nodes were further treated under sterile conditions on the clean bench. The nodes were incubated in 0.2% mercury chloride with one drop of Tween per 50 ml liquid (reduction of the surface tension) for 7 and 10 min. The experiment was conducted with 15 replications for each variant, including a non-sterilized control variant. Afterwards, the nodes were

washed three times with sterile water and transferred to sample tubes with 20 ml MS medium with 8 g/l agar. The survival rate of the nodes was determined over a period of seven weeks. In addition to determine the time required to successfully disinfect nodes, it was analyzed whether different ecotypes have a different susceptibility to pathogen infections following the disinfection procedure.

*M. oleifera* seeds originating from open land collection in Kolkata (India) were used for a second disinfection experiment. Two month after sowing, nodes from plants cultivated in soil filled pots were harvested and disinfected initially with 70% ethanol for 1 min, followed by the disinfection with 0.2% mercury chloride for 3, 5, or 7 min (20 replications). The nodes were washed in sterile water and transferred in sample tubes with 20 ml MS medium with 8 g/l agar. Over a period of five weeks, the infection rate of the nodes with germs was determined.

**_In vitro_ multiplication, growth, and callus formation from nodes under different growing conditions**

To determine optimum *in vitro* culture conditions and to establish callus cell cultures, the sterilized nodes (*M. oleifera* ecotype from India) were cultivated *in vitro* under different conditions. 1.5 to 2 cm long nodes of the previously *in vitro* established plants were used for the experiments. *In vitro* multiplication, growth, and callus formation of nodes from different plant parts in medium with varying agar and phytohormone concentration were analyzed.

In a first experiment the influence of BAP concentration on the main shoot height, the number and height of offshoots, and the plant multiplication rate were analyzed. Nodes were cultivated on MS medium with different agar contents (6 or 8 g/l) and BAP concentrations (0, 0.5, 0.75, 1 mg/l). After three weeks of cultivation, the growth parameters of the *in vitro* plants were determined. Five replications were carried out for each variant.

In a further experiment the base, middle, head parts, and offshoots of the mother plants, generated from the disinfected nodes of the *M. oleifera* ecotype from India, were isolated and cultivated on MS medium with 0.5 mg/l BAP and 8 g/l agar. One of the plant parts

shown in Figure 1 (base part, middle part head part, and off-shoot) were added to each flask containing 50 ml medium. Shoot multiplication and growth were investigated three weeks after cultivating. The experiment was performed in 10 replications. The quantity of newly developed middle parts, head parts, and offshoots was documented.

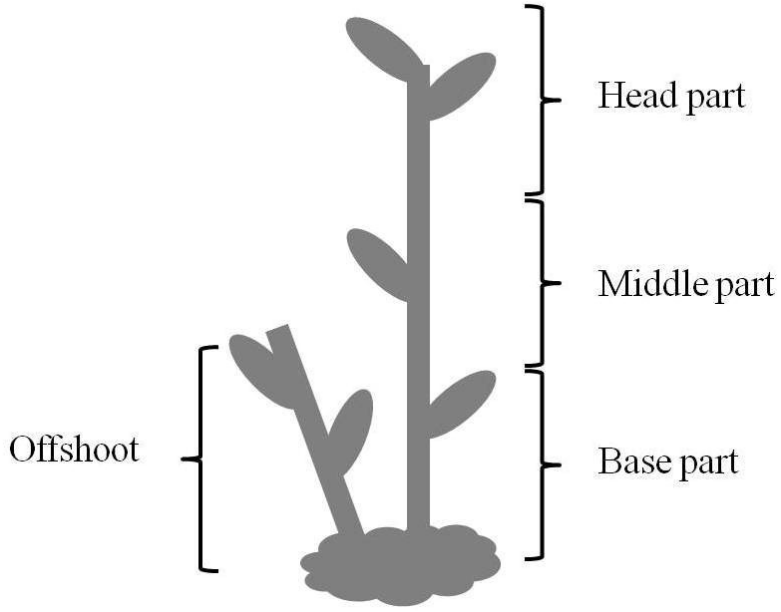



Fig. 1: Plant parts of *Moringa oleifera in vitro* plants

The induction of callus formation from nodes was investigated in 10 replications. Nodes (Indian ecotype) were cultivated on MS medium with different BAP concentrations (0, 0.5, 0.75, 1 mg/l) and agar contents (6 and 8 g/l agar) *in vitro*. The callus volume was determined after three weeks of cultivation *in vitro*.

Sterile leaves from *in vitro* cultivated *M. oleifera* plants generated from the disinfected notes (Indian ecotype) were used to study the effect of leaf orientation on cell differentiation and callus development. The leaves were grown on MS medium (half strength, 8 g/l agar) in an adaxial and abaxial direction. Plant leaves of each orientation were placed on plates with MS medium with different phytohormone concentrations. The medium was supplemented with two concentrations of IAA and TDZ. The first MS medium contained 1 mg/l IAA and 1 mg/l TDZ; the second contained twice the amount of both hormones. Callus parts derived from the adaxial and abaxial orientated leaves in the different phytohormone concentrations were further cultivated under the same

growing conditions. A volume of about 0.125 $cm^3$ (0.5 x 0.5 x 0.5 cm) of each variant was cultivated on plates. The callus was cultivated under light conditions or in the dark. All plates were light induced for 24 hours at the start of the experiment. Callus volume was measured after four weeks. The experiment was repeated three times with eight replications.

**Analysis of the glucosinolate content**

20 mg of freeze-dried, grounded *M. oleifera* leaf material was used for glucosinolate extraction. For the first extraction 750 µl of 70% methanol and 100 µl of a sinigrin standard solution (1 mM, internal standard, sinigrin hydrate from horseradish, Sigma-Aldrich) were added to the sample. The samples were extracted at 80 °C in a thermomixer for 10 min and then centrifuged at 6,700 g for 5 min to pellet the sample and to collect the supernatant. The pellet was twice re-extracted using 500 µl of 70% methanol for 5 min and centrifuged. Glucosinolates of the combined supernatants were desulfatized based on a modification of DIN EN ISO 9167-1:1995 and a method of Mewis et al. (2005). The extracts were analyzed on a Dionex P680A HPLC system equipped with an ASI-100 auto sampler and a PDA-100 photodiode array detector. Glucosinolates were separated on a narrow bore column (AcclaimTM 120 RP18:5 µm, 2.1 x 250 mm, Dionex) at 25 °C. Eluents used for HPLC analysis were: (a) 100% doubled distilled water and (b) 40% acetonitrile. The eluent flow program was: 0.5% B (0 - 1 min), 10% B (6 min), 20% B (15 min), 50% B (21 - 26 min), 99% B (29 - 33 min) and 0.5% B (36 - 43 min). The flow rate was 0.4 ml/min and the eluent was monitored at 229 nm. Chromeleon software Version 6.0 was used for quantitative peak evaluation. Quantitative analysis was based on the peak area relative to the internal standard sinigrin. The glucosinolates were identified by UV-spectrum, retention time and mass spectroscopy. 80 µl of the sample was injected into the HPLC for glucosinolate analysis.

**Statistical analysis**

Depending on the normality distribution of data, an ANOVA followed by a Tukey's HSD test or an ANOVA on ranks followed by a Tukey's HSD test were carried out to reveal statistical significances. The software SigmaPlot 12.3 was used for statistical analysis.

## Results

### Establishment of an *in vitro* culture to obtain sterile plant material

Due to the high degree of germ contamination of *M. oleifera* seeds, it was not possible to obtain a sterile culture from seeds using sodium hypochlorite. Contrary, mercury chloride as a disinfectant yields seeds without any visible infection with bacteria or fungi. However, seeds lost germination capacity following the use of either disinfectant. Therefore, we focused on other plant organs such as nodes in further experiments.

In the first disinfection test nodes from two Thai ecotypes without any visible germ contamination were observed using 0.2% mercury chloride (Figure 2). It was not possible to achieve sterile vital nodes without the use of the disinfectant mercury chloride. Seven weeks after using mercury chloride, 30% of the nodes remained non-infested with germs. No significant effect on the survival rate was found when the application time of mercury chloride was increased from 7 to 10 min. All sterile nodes were subsequently able to bud. No significant differences in the survival rate between the two analyzed ecotypes were found.

Based on the first experiment, a second disinfection experiment was carried out using nodes derived from the Indian ecotype. Infestation with germs was detected for all incubation times in 0.2% mercury chloride (Figure 3). Following germ infection the plant material failed to bud and died due to the infection. This was not attributed to the mercury chloride treatment. The disinfection test showed that the number of sterile vital nodes increased when treated for longer times with mercury chloride (3, 5, 7 min). Most of the nodes were infested with pathogens two weeks after disinfection. Following a mercury chloride treatment from 3 to 5 min, the rate of non-infected nodes was only 5% after 20 days. As shown in Figure 3, all nodes died within 23 days as a consequence of pathogen infection after a mercury chloride treatment of 3 min. Only one sterile node was achieved following a mercury chloride treatment of 5 min. The mercury chloride disinfection variant with an application time of 7 min yielded the best results with a maximum survival of non-pathogen infected nodes of 15%. Also the nodes were able to bud.

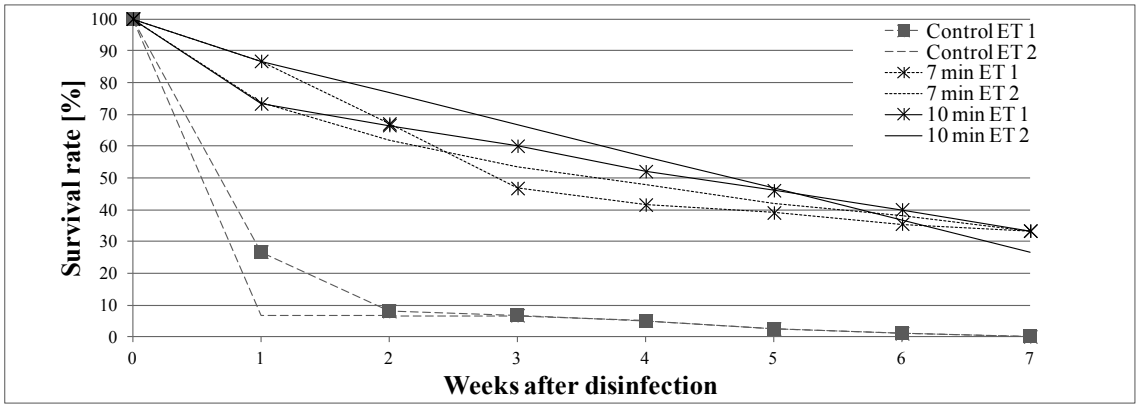



Fig. 2: Rate of non-pathogen-infected nodes of two *Moringa oleifera* ecotypes (ET 1, ET 2) from Thailand following disinfection with $HgCl_2$ (0.2%)

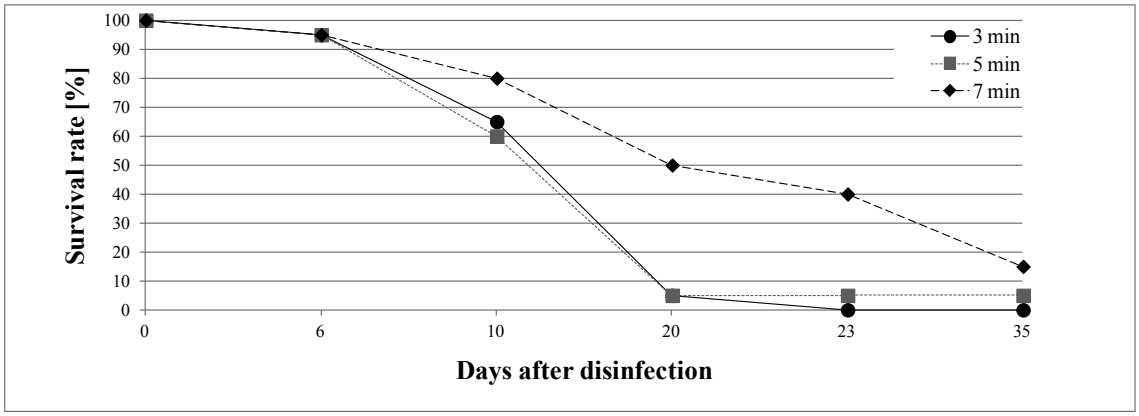



Fig. 3: Rate of non-pathogen-infected nodes of *Moringa oleifera* (Indian ecotype) after a different disinfection time with $HgCl_2$ (0.2%)

**Optimization of the *in vitro* cultivation of *M. oleifera***

The influence of different BAP and agar concentrations in MS medium on the main shoot height, the number and height of offshoots, and root development was investigated (Table 1, Figure 4). In the experiment with different BAP concentrations, the main shoots were significantly taller in the MS medium (6 and 8 g/l agar) without BAP application compared to medium with 1 mg/l BAP (Table 1). In media with 6 and 8 g/l agar concentration, the highest significant amounts of offshoots were detected in the medium with 0.5 mg/l BAP compared to medium with 0.75 mg/l BAP. The influence of the hormone concentration on offshoot height was only significant in medium with 6 g/l agar. When no phytohormones were applied, offshoots were significantly taller than those in

medium with BAP. The influence of the BAP and agar concentration on the growth habitus of *M. oleifera* is shown in Figure 4. The height of the plants' main shoot and offshoots decreased with an increased BAP concentration. Furthermore, shoot multiplication increased. The highest rate of shoot multiplication, with a 4.4-fold increase, was found in MS medium with 0.5 mg/l BAP.

Tab. 1: Characteristics of *Moringa oleifera* growth (height and number of shoots, root development) *in vitro* with different concentrations of BAP and agar in MS medium

| | **Agar concen-tration [g/l]** | **BAP concentration [mg/l]** | | | |
|---|---|---|---|---|---|
| | | **0** | **0.5** | **0.75** | **1** |
| **Main shoot height [cm]** | **6** | 4.10 (a) | 2.83 (a) | 1.60 (ab) | 1.15 (b) |
| | **8** | 3.57 (A) | 2.74 (B) | 1.63 (B) | 1.38 (B) |
| **Number of offshoots [n]** | **6** | 3.40 (ab) | 4.34 (a) | 2.47 (b) | 2.93 (b) |
| | **8** | 1.86 (B) | 4.40 (A) | 2.13 (B) | 3.53 (AB) |
| **Height of shoots [cm]** | **6** | 3.81 (a) | 2.21 (b) | 1.66 (bc) | 1.12 (c) |
| | **8** | 2.81 (A) | 2.63 (A) | 1.45 (A) | 1.61 (A) |
| **Root development rate [%]** | **6** | 60 (a) | 0 (b) | 0 (b) | 0 (b) |
| | **8** | 60 (A) | 0 (B) | 0 (B) | 0 (B) |

Different letters indicate significant differences in the height of the main shoot and the height and number of offshoots between different BAP concentrations, one-way ANOVA, Tukey's HSD test, $p < 0.05$ (minuscles: 6 g/l agar, capital letters: 8 g/l agar)

A spontaneous root development was detected in MS medium without BAP. The root developed about three weeks after placing plant nodes on MS medium without BAP. The *in vitro* plants were transferred to fresh MS medium without BAP every three weeks. The root development rate increased steadily and already nine weeks after transfer to BAP-free MS medium 60% of the plants (6 and 8 g/l agar) had formed roots (Table 1). Twelve weeks later, 100% of the plants *in vitro* possessed roots.

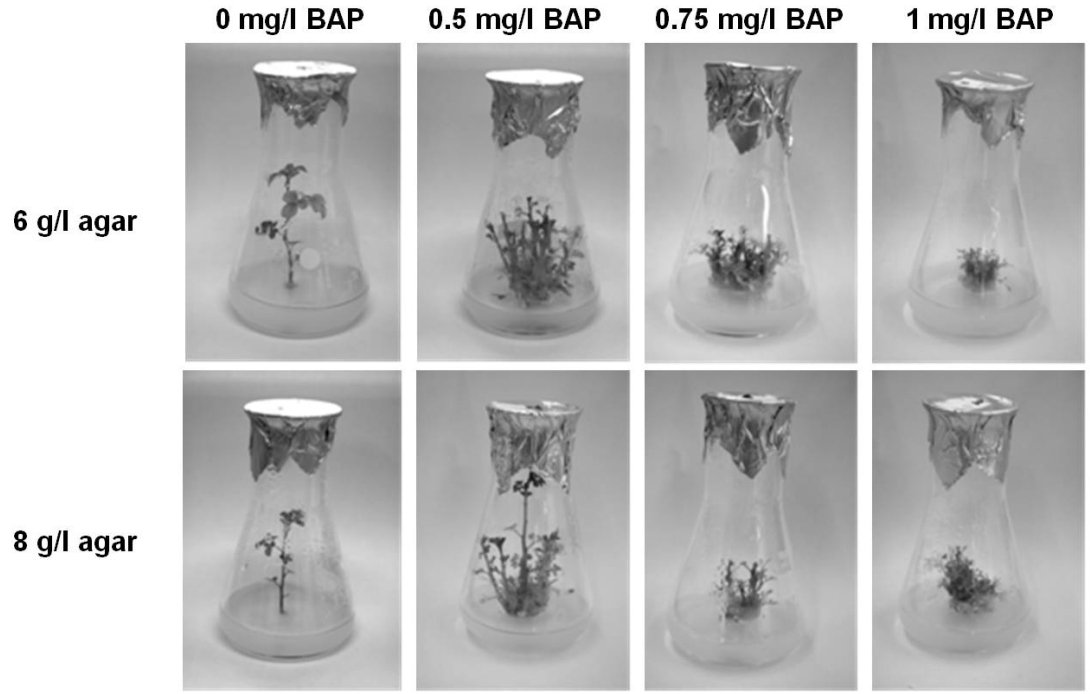



Fig. 4: Influence of BAP and agar concentration on the growth habitus of *Moringa oleifera in vitro*

**Multiplication ability of different plant parts**

The multiplication ability of different plant parts: base parts, middle parts, head parts, and offshoots on MS medium was studied (Figure 1 and 5, Table 2). The two tests revealed that after three weeks of cultivation, the base parts were the most suitable and most augmentable plant parts. The base parts formed an average of 7.3 (Table 2a) and 7.9 (Table 2b) multiplications. Head parts formed on average 4.9 multiplications after three weeks of cultivations on MS medium with 0.5 mg/l BAP and 8 g/l agar. Regardless of the initially used plant part used for the multiplication experiment, significantly smaller amounts of middle parts (0.7 and 0.6) and significantly larger amounts of head parts (2.4 and 1.8) and offshoots (1.9 and 2.9) developed. 6.1 and 6.3 multiplications were generated on average after three weeks of cultivation.

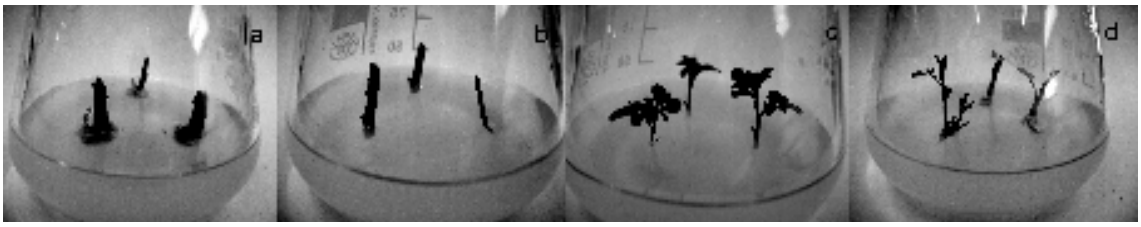



Fig. 5: Different *Moringa oleifera* plant parts established *in vitro,* a) base part, b) middle part, c) head part, d) offshoot

Tab. 2: The averaged number of plant parts formed from base parts, middle parts, head parts and offshoots of *Moringa oleifera* (repeated experiment, a and b)

a

| **Plant parts used** | **Averaged number of plant parts formed [n]** | | | | |
|---|---|---|---|---|---|
| | **B** | **M** | **H** | **O** | **Σ** |
| **Base part (B)** | 1.0 (b) ±0 | 1.2 (b) ±1.23 | 3.4 (a) ±1.43 | 1.7 (ab) ±1.70 | **7.3** |
| **Middle part (M)** | 1.0 (ab) ±0 | 0.5 (b) ±0.53 | 2.4 (a) ±1.35 | 2.4 (a) ±1.90 | **6.3** |
| **Head part (H)** | 1.0 (ab) ±0 | 0.5 (b) ±0.53 | 1.9 (a) ±0.88 | 1.5 (ab) ±1.27 | **4.9** |
| **Offshoot (O)** | 1.0 (a) ±0 | 0.7 (a) ±0.67 | 1.9 (a) ±1.37 | 2.1 (a) ±2.18 | **5.7** |
| **Ø** | **1.00 (BC)** | **0.73 (C)** | **2.40 (A)** | **1.93 (AB)** | **6.05** |

b

| **Plant parts used** | **Averaged number of plant parts formed [n]** | | | | |
|---|---|---|---|---|---|
| | **B** | **M** | **H** | **O** | **Σ** |
| **Base part (B)** | 1.0 (ab) ±0 | 0.6 (b) ±1.26 | 2.6 (a) ±1.65 | 3.7 (a) ±2.79 | **7.9** |
| **Middle part (M)** | 1.0 (ab) ±0 | 0.8 (b) ±1.48 | 1.6 (ab) ±1.65 | 3.4 (a) ±3.13 | **6.8** |
| **Head part (H)** | 1.0 (ab) ±0 | 0.5 (b) ±0.71 | 1.7 (a) ±0.95 | 1.7 (ab) ±1.70 | **4.9** |
| **Offshoot (O)** | 1.0 (ab) ±0 | 0.3 (b) ±0.67 | 1.4 (ab) ±1.07 | 2.8 (a) ±1.93 | **5.5** |
| **Ø** | **1.00 (B)** | **0.55 (C)** | **1.83 (AB)** | **2.90 (A)** | **6.28** |

Different letters indicate significant differences in the amount of plant parts formed derived from a certain plant part of *Moringa oleifera*, one-way Analysis on Ranks, Tukey's HSD test, $p < 0.05$

**Callus induction and formation of *in vitro M. oleifera* plant material**

The influence of BAP and agar concentration in the MS medium on callus formation was studied (Table 3). When no BAP was added, callus induction was significantly lower than in all other variants with added BAP. This was found for plants on MS medium with 6 g/l agar as well as for plants on MS medium with 8 g/l agar. No statistically significant differences were found between the two agar concentrations for nodes on MS medium with the same BAP concentration except for a BAP concentration of 0.75 mg/l.

Tab. 3: Callus formation [$cm^3$] of *Moringa oleifera* nodes on MS medium with different BAP and agar concentrations

| **Agar amount** | **BAP concentration [mg/l]** | | | |
|---|---|---|---|---|
| **[g/l]** | **0** | **0.5** | **0.75** | **1** |
| **6** | 0.22 (c) | 2.65 (ab) | 1.99 (b) | 3.80 (a) |
| **8** | 0.13 (c) | 1.66 (b) | 2.89 (a) | 2.77 (a) |

Different letters indicate significant differences in callus formation between different BAP concentrations, ANOVA on Ranks, Tukey's HSD test, $p < 0.01$

Furthermore, the effect of the phytohormones IAA and TDZ, light exposure, and leaf orientation on callus development were studied (Table 4, Figure 6). All callus parts, obtained from the further adaxial and abaxial orientated cultivated leaves, were placed on plates with a half strength MS medium (three each) with an initial volume of 0.5 x 0.5 x 0.5 cm. When cultivating with light exposure significantly smaller callus volumes were recorded. Under dark conditions an approximately 32-fold increase was observed after four weeks while the callus volume under light conditions was significantly lower with an approximately 2-fold increase (ANOVA on Ranks, Tukey's HSD test, $p < 0.05$). Neither the hormone concentrations of IAA and TDZ nor the leaf surface direction had any statistical impact on callus production.

Tab. 4: Callus volume and percentage increase in volume of *Moringa oleifera* callus with different hormone concentrations (IAA, TDZ), light exposure and leaf orientation

| Surface of the leaf | | Light IAA/TDZ concentration [mg/l] | | Dark IAA/TDZ concentration [mg/l] | |
|---|---|---|---|---|---|
| | | 1/1 | 2/2 | 1/1 | 2/2 |
| Adaxial | Callus volume [$cm^3$] | 0.15 | 0.14 | 4.18 | 3.50 |
| | Volume increase [%] | 22.22 | 13.33 | 3246.40 | 2703.82 |
| | Standard deviation | ±0.03 | ±0 | ±1.58 | ±1.26 |
| Abaxial | Callus volume [$cm^3$] | 0.48 | 0.19 | 4.11 | 4.17 |
| | Volume increase [%] | 283.47 | 53.69 | 3189.16 | 3238.67 |
| | Standard deviation | ±0.54 | ±0.12 | ±0.88 | ±0.44 |

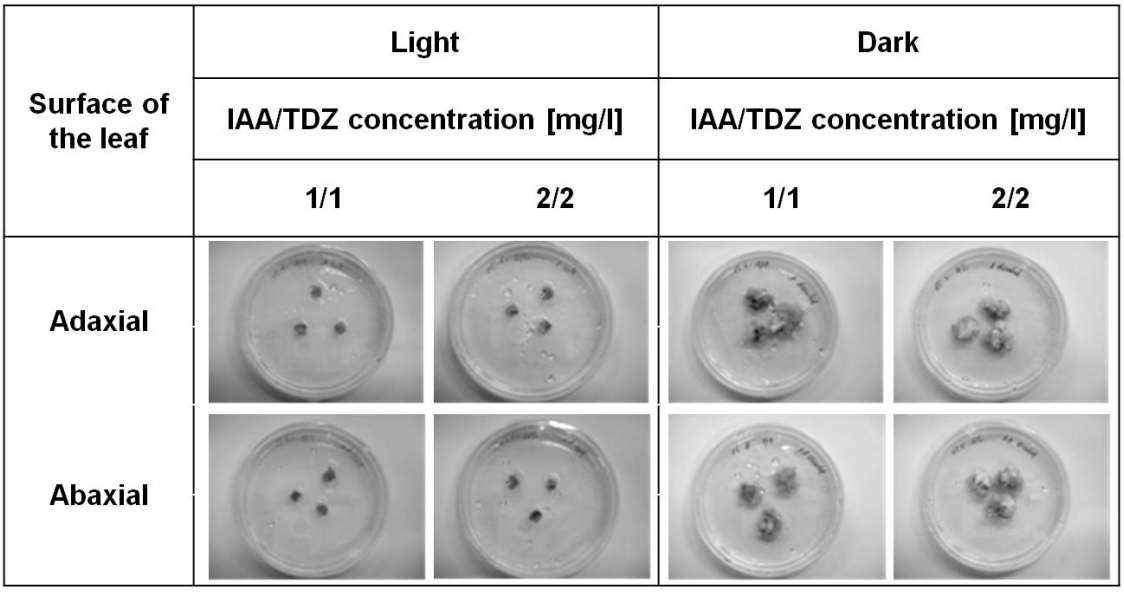



Fig. 6: Influence of different IAA and TDZ concentrations in ½ MS medium on callus growth of *Moringa oleifera*

**Glucosinolate content in leaves of *M. oleifera in vitro* material**

The glucosinolate content of *M. oleifera in vitro* cultures varied greatly from 5 to 130 µmol/g dry weight. The glucosinolate composition of *in vitro* material and greenhouse leaf material were completely different (Figure 7). Only rhamnopyranosyloxy-benzyl glucosinolates were detected in the greenhouse plants (Figure 7a). In the *in vitro* material, the main glucosinolate was identified as benzyl glucosinolate (Figure 7b), which was not found in the leaf material harvested from plants in the greenhouse.

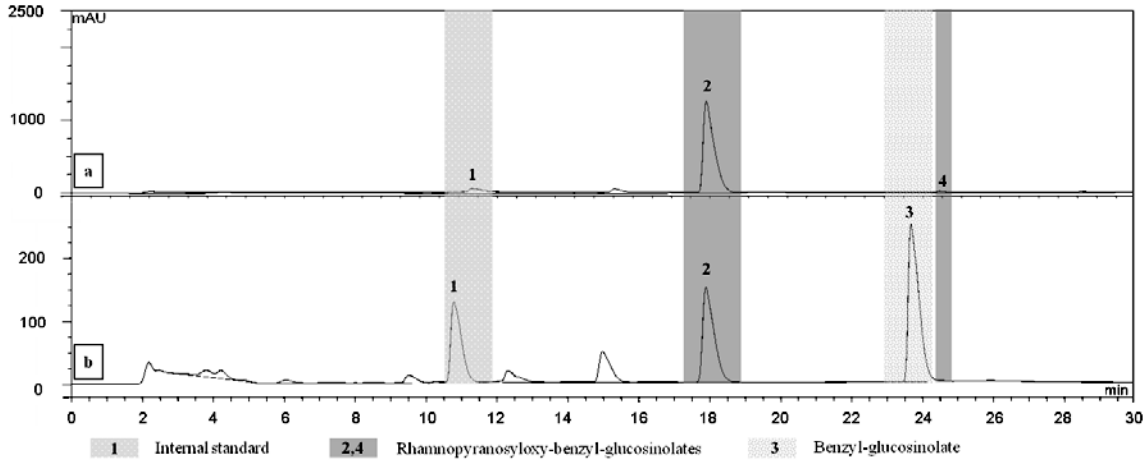



Fig. 7: Chromatograms of *Moringa oleifera* leaves, a) greenhouse leaves, b) *in vitro* leaves

## Discussion

Due to the frequent germ infestation of seeds, it was very difficult to establish a sterile *in vitro* culture of *M. oleifera* ecotypes. Seed-borne bacteria had already been mentioned by Steinitz et al. (2009). In contrast to Steinitz et al. (2009), no sterility was obtained after using sodium hypochlorite (NaOCl) for surface area disinfection in our initial experiments. This suggests that the germ infection of the different plant parts of *M. oleifera* was much higher in the material of the cultivars used. Therefore, it was not possible to kill all pathogens with NaOCl. However, mercury chloride - another disinfectant - showed more disinfection capacity. Sterile plant nodes, which were able to bud, were achieved this way. Mercury chloride had already been used by other authors in plant disinfection procedures (Faisal et al., 2005; Meyer & van Staden, 1995; Verma &

Singh, 2007). However, it was not possible to establish a sterile *in vitro* culture from seeds even when mercury chloride was used. Therefore, in line with a report of Stephenson & Fahey (2004), nodes were used for ongoing disinfection experiments to establish *M. oleifera in vitro*. The optimum treatment time for 0.2% mercury chloride was identified to be 7 min. Most nodes were sterilized within this time. The optimum sterilization time can differ from plant species to plant species. To disinfect petiole explants from *Tylophora indica,* for example, 0.1% $HgCl_2$ was used for 3 min (Faisal et al., 2005), while shoots from *Oxalis lineraris* were disinfected for 10 min with 0.2% $HgCl_2$ (Meyer & van Staden, 1995). To obtain sterile *in vitro* cultures of *Brassica campestris*, a cruciferous vegetable of the same order (Brassicales) as *M. oleifera*, 0.1% $HgCl_2$ was used for 5 to 7 min.

After the establishment of the *in vitro* culture of *M. oleifera* nodes, a successful propagation of the plant was achieved very quickly in the present study. Very high multiplication ability (six multiplications on average) was detectable over a cultivation period of three weeks (Table 2). Compared to the growth of other *in vitro* plants, the shoots and growth development of the *M. oleifera* plants was quite good. Nodal segments of *Macadamia* spp., for example, produced only 2 to 3 shoots within 4 to 6 weeks (Gitonga et al., 2010). The cultivation of axillary shoots from different chestnut clones for four weeks also led to the production of only 2 to 3 new shoots (Ballester et al., 2001).

Different plant parts were used to analyze the multiplication ability of *in vitro M. oleifera* cultures in MS medium with 0.5 mg/l BAP (Table 2). The present study revealed that especially the base parts of the *M. oleifera* plants have an enormous regeneration capacity, followed by middle parts and offshoots. From all the initial plant parts used, head parts and offshoots were produced much more frequently on MS than middle parts (Table 2). The *in vitro* plant growth and multiplication rate was analyzed adding different BAP concentrations to the MS medium (Table 1). When 0.5 mg/l BAP was added to the MS medium, plants growth was compressed and they had a greater ability to develop viable offshoots and other multiplications such as head parts. Plants in MS medium (8 g/l agar) without BAP were taller compared to medium containing BAP (Table 1). Head wilt was very often found in all plant parts in BAP-free medium. Concluding, it seems to be feasible to use base parts, offshoots, and head parts of *in vitro M. oleifera* plants to

propagate a plant population quickly. Definitely, plants should be cultivated on MS medium supplemented with 0.5 mg/l BAP. A spontaneous root development was detected in BAP-free MS medium. This suggests that formation of root development in *M. oleifera* plants can be easily elicitated. Further experiments should be conducted to test the effect of different auxins on *M. oleifera* root developments.

Since the glucosinolates in *M. oleifera* are of particular interest, the existence of these secondary metabolites in *in vitro* plants and changing contents of these ingredients at different supply steps of the plants should be further investigated. For example, a plant treatment with methyl jasmonate can enhance glucosinolate levels. Methyl jasmonate was used in a previous experiment by Doughty et al. (1995) to induce the glucosinolates in the leaves of oilseed rape. First analyses of glucosinolates in *M. oleifera in vitro* material (data not shown) showed that the glucosinolate content ranged from 5 to 130 µmol/g dry weight, depending on the medium composition, as well as on plant and callus development. The glucosinolates profile of leaf material from the greenhouse and the *in vitro* culture differ remarkably. The main glucosinolates of the leaves from greenhouse cultivation were rhamnopyranosyloxy-benzyl glucosinolates, whereas benzyl glucosinolate, the precursor of the rhamnopyranosyloxy-benzyl glucosinolate, was abundant in *in vitro* leaf material.

In further experiments, the influence of different nutrient compositions of the medium on growth and the production of secondary metabolites need to be analyzed. It is known from the literature that a specific sulfur application in connection with a sufficient nitrogen supply in open land cultivations of different plants increases the content of glucosinolates. In experiments with canola, Ahmad et al. (2007) showed that the glucosinolate content increased as the sulfur rate increased. In a greenhouse cultivation experiment with different clones of *M. oleifera*, an additional sulfur application to the plants led to a significant lower biomass production and increased the glucosinolate concentration in the leaves (Förster et al., 2011). Large-scale, complex open land experiments to analyze the influence of nutrition on the concentrations of glucosinolates in plant leaves can be avoided by cultivating *M. oleifera in vitro*. Furthermore, it would be possible to conduct preliminary experiments in nutrition combinations with much less effort.

The establishment of a callus culture could play a central role in upcoming and ongoing experiments. Callus is undifferentiated plant tissue that is able to produce glucosinolates. These compounds may accumulate under different conditions. The *in vitro* production of anthocyanin in callus cultures has been reported for *Oxalis linearis* (Meyer & van Staden, 1995). For this reason, it was important to obtain a medium that guarantees optimum callus production in its configuration. MS medium with BAP resulted in significantly higher callus production than media without this phytohormone. Although callus formation was found in MS medium with BAP, the use of IAA and TDZ was considered reasonable. The auxin-cytokinin ratio in the medium determines whether the plant tissue remains as an undifferentiated callus or forms shoots, roots, etc. Callus formation is supported with an equal ratio (Slater et al., 2003). Optimum callus production was obtained by placing a callus part in a half-strength MS medium with 1 mg/l IAA and 1 mg/l TDZ. In comparison to analysis of Liu & Cantliffe (1984), leaf orientation (abaxial or adaxal direction) had no influence on the callus formation in the conducted experiments. A significant increased callus formation was found when the callus was cultivated under dark condition. Some researchers report that different light conditions have an impact on callus formation (Smith et al., 1977; Omidi & Shahpri, 2003). In half strength MS medium supplemented with an equal concentration of IAA and TDZ callus formation was much more successful under dark conditions than on plants in MS medium with BAP. Tests analyzing callus formation in MS medium with BAP under dark conditions yield less callus production than the medium with IAA and TDZ (data not shown). Further experiments need to be conducted to analyze the concentration of glucosinolates in the callus of *M. oleifera*. It was possible to create a suspension culture using the callus obtained from the established callus culture. The enrichment of secondary metabolites in suspension cultures has been shown in many experiments. Pilgrim (1977) even reported the saponin concentrations in suspension cultures of *Digitalis purpurea* under different growth conditions.

The *in vitro* cultivation of *M. oleifera* is a promising possibility to obtain healthy plant material regardless of the season, weather and infestation. The availability of plant material all year round is essential to save time in ongoing experiments on analyzing the input of different growing conditions or stressors on the growth and accumulation of secondary metabolites in the plant. The *in vitro* growth of *M. oleifera* plants was much

faster than in the greenhouse. It takes just two weeks for a node to develop into an intact plant. A larger number of extensive experiments can be conducted within a much shorter period of time. Once the plant material had been sterilized, no re-infestation with germs was observed. For this reason, any influence of pathogens on the growth and secondary metabolism of the plants can be excluded in the experiments. Taking all the mentioned points into consideration, the *in vitro* culture of *M. oleifera* is a great opportunity for gaining fundamental knowledge on optimum cultivation to obtain plant material for different purposes and requirements.

## References

Ahmad, G.; Jan, A.; Arif, M.; Jan, M. T. & Khattak, R. A. (2007): Influence of nitrogen and sulfur fertilization on quality of canola (*Brassica napus* L.) under rainfed conditions. *Journal of Zhejiang University* SCIENCE *B* 8: 731-737.

Anwar, F.; Latif, S.; Ashraf, M. & Gilani, A. H. (2007): *Moringa oleifera*: A food plant with multiple medicinal uses. *Phytotherapy Research* 21: 17-25.

Ballester, A.; Bourrain, L.; Corredoira, E.; Goncalves, J. C.; Lè, C. L.; Miranda-Fontaiña, M. E.; San-José, M. del C.; Sauer, U.; Vieitez, A. M. & Wilhelm, E. (2001): Improving chestnut micropropagation through axillary shoot development and somatic embryogenesis. *Forest Snow and Landscape Research* 76: 460-467.

Bennett, R. N.; Mellon, F. A.; Foidl, N.; Pratt, J. H.; Dupont, M. S.; Perkins, L. & Kroon, P. A. (2003): Profiling glucosinolates and phenolics in vegetative and reproductive tissues of the multi-purpose trees *Moringa oleifera* L. (Horseradish Tree) and *Moringa stenopetala* L. *Journal of Agricultural and Food Chemistry* 51: 3546-3553.

Cáceres, A.; Saravia, A.; Rizzo, S.; Zabala, L.; De Leon, E. & Nave, F. (1992): Pharmacologic properties of *Moringa oleifera*. 2: Screening for antispasmodic, anti-inflammatory and diuretic activity. *Journal of Ethnopharmacology* 36: 233-237.

Decendit, A. & Mérillon, J. M. (1996): Condensed tannin and anthocyanin production in *Vitis vinifera* cell suspension cultures. *Plant Cell Reports* 15: 762-765.

Doughty, K. J.; Kiddle, G. A.; Pye, B. J.; Wallsgrove, R. M. & Pickett, J. A, (1995): Selective induction of glucosinolates in oilseed rape leaves by methyl jasmonate. *Phytochemistry* 38: 347-350.

Eilert, U., Wolters, B. & Nahrstedt, A. (1981): The antibiotic principle of seeds of *Moringa oleifera* and *Moringa stenopetala*. *Planta Medica* 42: 55-61.

Fahey, J. W. (2005): *Moringa oleifera*: A Review of the medical evidence for its nutritional, therapeutic, and prophylactic properties. Part 1. *Trees for Life Journal* (http://www.tfljournal.org/article.php/20051201124931586), (Zugriff 8.5.2017).

Faisal, M., Singh, S. & Anis, M. (2005): *In vitro* regeneration and plant establishment of *Tylophora indica* (Burm. F.) Merrill: petiole callus culture. *In Vitro Cellular & Developmental Biology - Plant* 41: 511-515.

Faizi, S.; Siddiqui, B. S.; Saleem, R.; Siddiqui, S.; Aftab, K. & Gilani, A. H. (1994): Isolation and structure elucidation of new nitrile and mustard oil glycosides from *Moringa oleifera* and their effect on blood pressure. *Journal of Natural Products* 57: 1256-1261.

Förster, N., Mewis, I. & Ulrichs, C. (2011): Einfluss des Anbaus auf die Wachstumseigenschaften und den Glucosinolatgehalt von *Moringa oleifera*. *DGG-Proceedings* 1(14): 1-5.

Frankel, E. N., Kanner, J., German, J. B., Parks, E. & Kinsella, J. E. (1993): Inhibition of oxidation of human low-density lipoproteins by phenolic substances in red wine. *The Lancet* 341: 454-456.

Gilani, A. H.; Aftab, K. & Suria, A. (1994): Pharmacological studies on hypotensive and spasmolytic activities of pure compounds from *Moringa oleifera*. *Phytotherapy Research* 8: 87-91.

Gitonga, L. N.; Cichuki, S. T.; Namau, K.; Muigai, A. W, T.; Kahangi, E. M; Wasilwa, L. A.; Wepukhulu, S. & Njogu, N. (2010): Effect of explants type, source and genotype on *in vitro* shoot regeneration in Macadamia. *Journal of Agricultural Biotechnology and Sustainable Development* 2: 129-135.

Guevara, A. P.; Vargas, C.; Sakurai, H.; Fujiwara, Y.; Hahimoto, K.; Maoka, T.; Kozuka, M.; Ito, Y.; Tokuda, H. & Nishino, H. (1999): An antitumor promoter from *Moringa oleifera* Lam. *Mutation Research* 440: 181-188.

Liu, R. J. & Cantliffe, D. J. (1984): Somatic embryogenesis and plant regeneration in tissue cultures of sweet potato (*Ipomea batatas* Poir.). *Plant Cell Reports* 3: 112-115.

Makonnen, E.; Hunde, A. & Damecha, G. (1997): Hypoglycaemic effect of *Moringa stenopetala* aqueous extract in rabbits. *Phytotherapy Research* 11: 147-148.

Mewis, I.; Appel, H. M.; Hom, A.; Raina, R. & Schultz, J. C. (2005): Major signaling pathways modulate Arabidopsis glucosinolate accumulation and response to both phloem-feeding and chewing insects. *Plant Physiology* 138: 1149-1162.

Meyer, H. J. & Van Staden, J. (1995): The *in vitro* production of an anthocyanin from callus cultures of *Oxalis lineraris*. *Plant Cell, Tissue and Organ Culture* 40: 55-58.

Murashige, T. & Skoog, F. (1962): A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.

NA 057-05-05 AA - Joint committee of DIN and DGF for the analysis of fats, oils and products thereof, related and primary products. (2012): Rapeseed - Determination of glucosinolate content - Part 1: Method using high-performance liquid chromatography (ISO 9167-1:1992/DAM 1:2012), German version EN ISO 9167-1:1995/prA1:2012.

Nadkarni, K. M. (1976): Nadkarni's Indian Materia Medica. Popular Prakashan, Bombay.

Nikkon, F.; Saud, Z. A.; Rahman, M. H. & Haque, M. d. E. (2003): *In vitro* antimicrobial activity of the compound isolated from chloroform extract of *Moringa oleifera* Lam. *Pakistan Journal of Biological Sciences* 6: 1888-1890.

Olson, M. E. (2002): Combining data from DNA sequences and morphology for a phylogeny of Moringaceae (Brassicales). *Systematic Botany* 27: 55-73.

Omidi, M. & Shahpiri, A. (2003): Callus induction and plant regeneration *in vitro* in potato. *Acta Horticulturae (ISHS)* 619: 315-322.

Pilgrim, H. (1977): Sapogeninbildung in Suspensionskulturen von *Digitales purpurea*. *Phytochemistry* 16: 1311-1312.

Ramachandran, C.; Peter, K. V. & Gopalakrishnan, P. K. (1980): Drumstick (*Moringa oleifera*): A multipurpose Indian vegetable. *Economic Botany* 34: 276-283.

Sampson, W. (2005): Studying Herbal Remedies. *The New England Journal of Medicine* 353: 337-339.

Siddhuraju, P. & Becker, K. (2003): Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringa oleifera* Lam.) leaves. *Journal of Agricultural and Food Chemistry* 51: 2144-2155.

Slater, A.; Scott, N. & Fowler, M. (2003): Plant Biotechnology. The genetic manipulation of plants. Oxford University press, Oxford.

Smith, R. H.; Price, H. J. & Thaxton, J. B. (1977): Defined conditions for the initiation and growth of cotton callus *in vitro* I. *Gossypium arboretum*. *In Vitro* 13: 329-334.

Steinitz, B.; Tabib, Y.; Gaba, V.; Gefen, T. & Vaknin, Y. (2009): Vegetative micro-cloning to sustain biodiversity of threatened *Moringa* species. *In Vitro Cellular & Developmental Biology - Plant* 45: 65-71.

Stephenson, K. K. & Fahey, J. W. (2004): Development of tissue culture methods for the rescue and propagation of endangered *Moringa* spp. germplasm. *Economic Botany* 58 (Supplement): 116-124.

Sultana, B. & Anwar, F. (2008): Flavonols (kaempeferol, quercetin, myricetin) contents of selected fruits, vegetables and medicinal plants. *Food Chemistry* 108: 879-884.

Talalay, P. & Talalay, P (2001): The importance of using scientific principles in the development of medicinal agents from plants. *Academic Medicine* 76: 238-247.

Verma, R. & Singh, R. R. (2007): Regeneration and *in vitro* flowering in *Brassica campestris* (L.) var. Bhavani. *Our Nature* 5: 21-24.

Wielanek, M. & Urbanek, H. (1999): Glucotropeaolin and myrosinase production in hairy root cultures of *Tropaeolum majus*. *Plant Cell, Tissue and Organ Culture* 57: 39-45.

Yang, R.-Y.; Chang, L.-C.; Hsu, J.-C.; Weng, B. B. C.; Palada, M. C.; Chadha, M. L. & Levasseur, V. (2006): Nutritional and functional properties of *Moringa* leaves – from germplasm, to plant, to food, to health, Konferenz: *Moringa* and other highly nutritious plant resources: Strategies, standards and markets for a better impact on nutrition in Africa (16. – 18. November 2006, Accra, Ghana, Africa).

## Kapitel III - Detektion von *M. oleifera*-Glucosinolaten sowie Standardherstellung

Um Glucosinolate qualitativ und quantitativ zu bestimmen, werden die chemischen Verbindungen nach DIN EN ISO 9167-1:1995 (NA 057-05-05 AA) zunächst extrahiert und dann mittels Hochdruckflüssigchromatographie (HPLC) vermessen. Die bereits durch Bennett et al. (2003) identifizierten und beschriebenen Glucosinolate in den Blättern von *M. oleifera* konnten jedoch mit diesem standardisierten Extraktionsverfahren nicht detektiert werden. Die multi-glykosylierte aromatische Glucosinolatstruktur von *M. oleifera* konnte nicht nachgewiesen werden. Das vermessene Glucosinolatspektrum von *M. oleifera* entsprach folglich nach dem Desulfatisierungsprozess (DIN-Methode) nicht dem eigentlich bestehenden im Blatt.

Aufgrund von Studien zur Bioaktivität verschiedener Abbauprodukte von Glucosinolaten wie z. B. Sulforaphan und Benzyl-Isothiocyanat (u. a. Clarke et al., 2008; Xiao et al., 2006), liegt die Vermutung nahe, dass die medizinischen Wirksamkeiten der Pflanze auf die Glucosinolate in *M. oleifera* zurückgeführt werden können. Bis heute durchgeführte Studien wurden meist an methanolischen, ethanolischen sowie wässrigen Extrakten von Blatt, Wurzel und Schote durchgeführt (u. a. Bharali et al., 2003; Budda et al., 2011; Costa-Lotufo et al., 2005). Die im vorliegenden Dissertationsvorhaben durchgeführten Analysen zur potentiellen Bioaktivität von *M. oleifera* verwendeten reine Glucosinolatextrakte (Gesamtglucosinolatextrakt sowie Einzelglucosinolate). Um Glucosinolatstandards aus den Blättern von *M. oleifera* generieren zu können, müssen diese zunächst auf ihre Verwendbarkeit (Stabilität) geprüft werden.

Um die dargestellten Fragestellungen beantworten zu können, sollten folgende Sachverhalte untersucht werden:

- *Kann eine geeignete Extraktionsmethode entwickelt werden, welche die Glucosinolate in M. oleifera realistisch abbildet?*
- *Wie unterscheiden sich die Glucosinolatspektren zwischen der DIN-Methode und der neu erarbeiteten? Wie entstehen diese Unterschiede?*

- *Ist es möglich einzelne Glucosinolate aus dem Gesamtextrakt von M. oleifera zu fraktionieren? Können Glucosinolatstandards aus Blattmaterial hergestellt werden?*
- *Wie stabil sind die hergestellten Standards (Gesamtextrakt, 1. Hauptglucosinolat: 4-α-Rhamnopyranosyloxy-Benzylglucosinolat, 2. Hauptglucosinolat: Acetyl-4-α-Rhamnopyranosyloxy-Benzylglucosinolat Isomer III)?*
- *Welchen Umbau- und Abbauprozessen unterliegen die Standards?*
- *Wie sollten die Standards gelagert werden, um Degradations- und Umformungsprozesse zu vermeiden?*

Die in diesem Kapitel dargestellten Fragenkomplexe werden in folgender Publikation behandelt:

> Förster, N.; Ulrichs, C.; Schreiner, M.; Müller, C. T. & Mewis, I. (2015): Development of a reliable extraction and quantification method for glucosinolates in *Moringa oleifera*. *Food Chemistry* 166: 456-464.

## Abstract

Glucosinolates are the characteristic secondary metabolites of plants in the order Brassicales. To date the common DIN extraction 'desulfo glucosinolates' method remains the common procedure for determination and quantification of glucosinolates. However, the desulfation step in the extraction of glucosinolates from *Moringa oleifera* leaves resulted in complete conversion and degradation of the naturally occurring glucosinolates in this plant. Therefore, a method for extraction of intact *Moringa* glucosinolates was developed and no conversion and degradation of the different rhamnopyranosyloxy-benzyl glucosinolates was found. Buffered eluents (0.1 M ammonium acetate) were necessary to stabilize 4-α-rhamnopyranosyloxy-benzyl glucosinolate (Rhamno-Benzyl-GS) and acetyl-4-α-rhamnopyranosyloxy-benzyl glucosinolate isomers (Ac-Isomers-GS) during HPLC analysis. Due to the instability of intact *Moringa* glucosinolates at room temperature and during the purification process of single glucosinolates, influences of different storage (room temperature, frozen, thawing and refreezing) and buffer conditions on glucosinolate conversion were analyzed. Conversion and degradations processes were especially determined for the Ac-Isomers-GS III.

## Introduction

Glucosinolates are one of the most investigated groups of secondary metabolites in plants. They are of crucial importance in insect-plant interactions and can act as feeding deterrent (generalists) or as attractant (specialists) (Mewis et al., 2012). Additionally, glucosinolates have the ability to influence human health as phytopharmaceutical, nutraceutical, or as food product (Melchini et al., 2013; Prakash & Gupta, 2012). Glucosinolates are a group of secondary plant metabolites found almost exclusively in plants of the order Brassicales. Structurally glucosinolates are β-S-glucosides of thio-oxime-O-sulphates and synthetized from amino acids. They are commonly divided into aliphatic, indolic, and aromatic glucosinolates based on their side chains, which stem from the precursor amino acids methionine, tryptophan and phenylalanine respectively. Profile and abundance of glucosinolates vary between different parts of a plant. For example, roots of over 29 analyzed plant species (mainly members of the Brassicaceae) contained significantly higher aromatic glucosinolate levels than shoots (Dam et al.,

2009). Aromatic glucosinolates such as *p*-hydroxybenzyl glucosinolate (sinalbin), 2-phenylethyl glucosinolate (gluconasturtiin), benzyl glucosinolate (glucotropaeolin), or 2(R)-hydroxy-2-phenylethyl glucosinolate (glucobarbarin) dominated in seeds and roots of white mustard (*Sinapsis alba*), watercress (*Barbarea verna)*, Indian mustard (*Tropaeolum majus*), or winter cress (*Barbarea vulgaris*) (Bennett et al., 2004; Leur et al., 2006). The family Moringaceae (order Brassicales) includes only the genus *Moringa*. In contrast to other Brassicales, all species of *Moringa*, and particularly *Moringa oleifera,* contain very high amounts of aromatic glucosinolates in all parts of the plant including leaves and roots, which overall showed lower levels of glucosinolates than seeds (seeds around 200 mg/g dry weight (dw), young leaves around 116 mg/g dw, older leaves around 63 mg/g dw, roots around 43 mg/g dw, Bennett et al., 2003).

Structurally, all multiglycosylated aromatic glucosinolates contain a hydroxy-benzyl moiety with a sugar molecule - rhamnose or arabinose - linked glycosidically to the aromatic ring. This generally appears to be unique to species of the genera *Moringa, Hesperis*, and *Reseda* (Bennett et al., 2003; Fahey et al., 2001). 4-α-rhamnopyranosyloxy-benzyl glucosinolate (Rhamno-Benzyl-GS) has been identified as the dominant leaf glucosinolate of *M. oleifera* and is accompanied by lower levels of three isomeric acetyl-4-α-rhamnopyranosyloxy-benzyl glucosinolates (Ac-Isomer-GSs I, II, III), which reflect the three position of the acetyl group at the rhamnose moiety of the molecule (Bennett et al., 2003). These aromatic glucosinolates are present in methanolic or aqueous leaf extracts of *M. oleifera*, and have been linked to reported antimicrobial (Cáceres et al., 1991), anti-inflammatory (Cáceres et al., 1992), detoxifying (Singh & Kumar, 1999), and anticancerogenic properties (Guevara et al., 1999) of such extracts. However, it has not been investigated sufficiently yet whether such activity can be linked to a specific glucosinolate or mixtures thereof. Linking observed antioxidative or anticancerogenic effects to specific glucosinolates or their mixtures, requires first and foremost a reliable method for quantification of *M. oleifera* glucosinolates and subsequent preparation of individual glucosinolates of *M. oleifera*.

Currently, the prevalent method for determination of glucosinolates in plants is the ‘desulfo glucosinolate extraction’ method according to DIN EN ISO 9167-1:1995 (NA 057-05-05 AA). The method uses an anion exchange columns (DEAE Sephadex A25) to

retain glucosinolates from methanolic plant extracts. Bonded, intact glucosinolates are desulfated enzymatically with sulfatase (H1, *Helix pomatia*) and eluted desulfo derivatives are analyzed with HPLC. Preliminary experiments showed that glucosinolate profiles of leaf extracts of *M. oleifera* using the desulfo method did not match profiles using a non-desulfating approach. The main aim of this study was to develop a reliable method for determination of natural glucosinolate profiles in *M. oleifera*. Specifically, we compared the results of the desulfo glucosinolate extraction method with an extraction method yielding intact glucosinolates. Furthermore, we investigated in detail conversion and degradation processes of purified glucosinolates under different analytical and storage conditions (e. g. different HPLC eluents, storage time and temperature, solvent).

## Materials and methods

### Plant material

Seeds of *M. oleifera* (ecotype number: 4880, origin in the USA) were obtained from the "AVRDC - The World Vegetable Center" (Shanhua, Tainan, Taiwan) and cultivated in greenhouse facilities at the Leibniz-Institute of Vegetable and Ornamental Crops Großbeeren/Erfurt e.V. in Großbeeren, Germany. Trees were planted in a grid with 0.5 m distance of each other in a 1 m deep greenhouse bed. Drip irrigation and a nebulization system were used to humidify soil and air respectively. Mean temperature in the greenhouse was 18.6 °C and varied between 20.5 °C (August) and 14.6 °C (October) over the harvest time of *M. oleifera* from June to October 2011. Temperature of ventilation was set to 19 °C. Leaves from the top of the plants (upper 20 cm, youngest leaves) were harvested three times a year in July, September, and October, freeze dried (Christ beta 1-16, Germany), and stored in the dark at room temperature. This plant material was used for testing glucosinolate extraction procedures and for the preparation of different *Moringa* glucosinolate standards. The series of consecutive performed experiments is shown in the supplemental Figure 1 (Suppl. Figure 1) and are described in detail in the respective method section.

**Comparison of two extraction methods for glucosinolate analysis**

Desulfo glucosinolate extraction method and intact glucosinolate extraction method were used in order to investigate which method delivered a reliable determination of the natural glucosinolate profile of *M. oleifera*.

*Desulfo glucosinolate extraction method*

The desulfo glucosinolate extraction method is based on a modification of DIN EN ISO 9167-1:1995 (NA 057-05-05 AA; 2012) and a method of Mewis et al. (2005). 20 mg of the pulverized leaf material per sample was extracted with 750 µl of 70% methanol and 100 µl of 2-propenyl glucosinolate (1 mM, sinigrin hydrate from horseradish, Sigma-Aldrich) was added as internal standard. Samples were heated in a thermo-mixer at 80 °C for 10 min, centrifuged at 16,000 g (Thermo Scientific, Heraeus Megafuge 11R Centrifuge) for 10 min, and the supernatant was collected. The pellet was extracted twice more with 500 µl of 70% methanol for 5 min. Glucosinolates of the combined supernatants of each sample were loaded and subsequently desulfated on DEAE Sephadex A25 columns. For this purpose, 500 µl DEAE Sephadex (suspended in 2 M acetic acid, v/v: 1/2) was added to empty cartridges (BIORAD, 10 ml volume). Columns were conditioned prior to loading of extracts with 2 x 1 ml 6 M imidazole solution (in 30% formic acid) followed by 2 x 1 ml ultra-pure water (MilliQ quality). Methanolic glucosinolate extracts were loaded on preconditioned columns, washed with 2 x 1 ml 0.02 M sodium acetate buffer (pH 4.0), and treated with 75 µl of a cleaned-up sulfatase solution (see below). Loaded columns were sealed and left to incubate at room temperature overnight. The following day the columns were washed with 1 ml ultra-pure water (MilliQ quality) to elute the desulfo glucosinolates. Eluates were filtered using Costar® Spin-X tubes (0.22 µm, Corning), transferred to 2 ml HPLC vials, and stored at -20 °C until measurement. For preparing the sulfatase, 5 ml raw sulfatase (aryl sulfatase, Th. Geyer GmbH) was purified after a method of the Commission Regulation (EEC) of the European Economic Community (EEC No. 2435/86, 1986). The cleaned-up sulfatase solution was stored at -20 °C until usage.

*Intact glucosinolate extraction method*

A method for extraction of intact glucosinolates was developed based on Lewke et al. (1996). Plant material used for methanolic extraction was also 20 mg. The first extraction steps using three times 70% methanol (80 °C) and 100 µl 2-propenyl glucosinolate as internal standard were performed as described for the desulfo glucosinolate extraction method. Instead of loading the combined supernatants onto DEAE sephadex columns, the supernatants were concentrated in a vacuum concentrator (Thermo Scientific Savent SPD111V Concentrator, vacuum pump: Vacuumbrand PC 3000 series, SVC3000) to a volume of 150 µl. 200 µl barium acetate (0.4 M) were added to each tube and made up to 1 ml with ultra-pure water (MilliQ quality). Samples were incubated for 30 min at room temperature and centrifuged at 16,000 g (Thermo Scientific, Heraeus Megafuge 11R Centrifuge) for 10 min. Supernatants were decanted and made up to 2 ml with ultra-pure water. 1 ml of each sample was filtered using Costar® SpinX tubes. Filtrates were transferred to HPLC vials. The remaining amount of each sample (about 1 ml) was incubated with 0.05 U myrosinase (thioglucosidase, Sigma Aldrich) for 8 h at 37 °C to hydrolyze glucosinolates to their corresponding breakdown products. Hydrolyzed sample were filtered through a Costar® SpinX tube and transferred to HPLC sample vials.

*HPLC analysis of desulfo and intact glucosinolate extracts*

Extracts from desulfo and intact glucosinolate extraction method were qualitatively and quantitatively analyzed on Merck Hitachi HPLC system. 10 µl sample were injected (LaChrom L-7200 auto sampler) on a 4.6 x 250 mm SB-C18 column (Zorbax 5 µm, Agilent) and separated using the following gradient program: 0 - 2 min: 0 - 1% B, 2 - 20 min: 1 - 50% B, 20 - 24 min: 50 - 100% B, 24 - 26 min: 100% B, 26 - 27 min: 100 - 1% B, and 27 - 35 min: 1 - 0% B at a flow rate of 1.5 ml/min. Unbuffered eluents were used for desulfo extracts with solvent A: 100% water and solvent B: 40% acetonitrile/water and buffered eluents for intact extracts with solvent A: 100% 0.1 M ammonium acetate, B: 40% acetonitrile/0.1 M ammonium acetate. Detection was at 229 nm on a photodiode array detector (LaChrom L-7455), components were identified from retention time (intact and desulfo glucosinolates) and UV spectra (desulfo glucosinolates) and quantified against internal standard. Retention times were 11.1 min for *p*-hydroxybenzyl glucosinolate and 12.5 min for Rhamno-Benzyl-GS for the analysis of desulfo glucosinolates in unbuffered eluents and 10.1 min for Rhamno-Benzyl-GS,

13.7 min for Ac-Isomer-GS I, 14.8 min for Ac-Isomer-GS II, and 17.6 min for Ac-Isomer-GS III for the analysis of intact glucosinolates in buffered eluents. The peak area remaining after myrosinase treatment of the intact glucosinolate extract was subtracted from the peak area of the glucosinolate of the intact glucosinolate extract without myrosinase treatment. This way interfering/co-eluting ballast compounds in the extract were excluded and a correct determination of the glucosinolate content was possible. Relative response factors (RF) used to correct for absorbance difference between the reference standard 2-propenyl glucosinolate (RF = 1) and the glucosinolates found in the *M. oleifera* extracts were determined as 0.59 for *p*-hydroxybenzyl glucosinolate and 0.68 for Rhamno-Benzyl-GS and the three Ac-Isomer-GSs.

**Isolation of intact glucosinolates for stability testing**

*Preparation of purified glucosinolate mixture*

To produce sufficient starting material for the fractionation of individual glucosinolates an intact glucosinolate extraction method based on Thies (1988) was used. 25 g powdered freeze-dried leaf material was heated to 80 °C in 300 ml 70% methanol. After centrifugation at 12,000 g for 10 min (Heraeus SEPATECH, Superfuge 22) the pellet was re-extracted twice with 100 ml 70% methanol. Combined supernatants were loaded on DEAE Sephadex columns (Telos Filtration Columns, Kinesis, 150 ml). Columns were packed with 60 ml DEAE Sephadex solution (10 g DEAE Sephadex A25 (Sigma Aldrich) suspended in 100 ml 2 M acetic acid), rinsed twice with 40 ml 6 M imidazole solution (40 g imidazole in 100 ml 30% formic acid), and twice with 40 ml ultra-pure water before loading the plant extract. After drop-wise applications of 500 ml extract per column, columns were drop-wise washed three times with 20 ml of formic acid:isopropanol:water (ratio 3:2:5) followed by four times with 20 ml ultra-pure water (MilliQ quality). Pre-tests showed that it was necessary to give the glucosinolate extract drop-wise to the column. A flash flow of all chemicals promoted glucosinolate loss. Glucosinolates were slowly eluted with a total of 100 ml 0.5 M potassium sulfate and collected into 100 ml absolute ethanol (99.9%). After centrifugation at 12,000 g for 10 min (Heraeus SEPATECH, Superfuge 22), the supernatants were dried in 100 ml pear-shape flasks (starting with 50 ml per flask) in a rotary evaporator, at 45 °C (Heidolph VV 2000 rotary evaporator with bath WB 2000, vacuum pump: Vacuumbrand PC 3000 series, SVC3000).

Dried residues were re-dissolved in three successive volumes of 4 ml methanol (99.8%) aided by sonification, and solutions were centrifuged for 10 min at 10,000 g (Thermo Scientific, Heraeus Megafuge 11R Centrifuge). 36 ml absolute ethanol (99.9%) were added to the supernatants and solvents were evaporated to dryness in 100 ml pear-shape flasks on a rotary evaporator at 45 °C (same as above). The resulting crystallized glucosinolate mixture was re-dissolved in 4 ml ultra-pure water and used as *M. oleifera* total glucosinolate standard and for fractionation into single glucosinolates.

*Preparation of single glucosinolates*

Purified glucosinolate standards were fractionated with a Dionex HPLC system equipped with a fraction collector (Dionex FOXY). 10 µl of the total glucosinolate standard from *M. oleifera* were injected into a Dionex P680A HPLC system equipped with an ASI-100 auto sampler and a U3000 photodiode array detector. Glucosinolates were separated on 2.1 x 250 mm AcclaimTM 120 RP18 column (5 µm, Dionex) at 25 °C. The eluent program was: 0 - 1 min 0.5% B, 8 - 10 min 20% B, 18 - 19 min 50% B, 28 - 33 min 99% B and 36 - 43 min 0.5% B at a flow rate of 0.4 ml/min and the eluent was monitored at 229 nm. Depending on the experiment described in the following method section, unbuffered or buffered eluents as described previously for extracts were used for HPLC analysis fractionation. Retention times of intact glucosinolates depended on the eluents and were 13.4 min/10.8 min in unbuffered/buffered eluents (u/b) for Rhamno-Benzyl-GS, 18.6 min/14.2 min u/b for Ac-Isomer-GS I, 19.5 min/15.5 min u/b for Ac-Isomer-GS II, and 22.1 min/18.6 min u/b for Ac-Isomer-GS III. Single peaks were collected at these times by the fraction collector. Fractions were dried in a vacuum evaporator, dissolved in 1 ml ultra-pure water or buffer (0.1 M ammonium acetate), and analyzed mass spectrometrically to confirm identity of individual glucosinolates, (section: 2.5). Purified individual glucosinolates were used to determine the response factors relative to 2-propenyl glucosinolate. The glucosinolate content was determined using 2-propenyl glucosinolate as internal standard.

**Stability testing of total glucosinolate standard and isolates**

The stability of the purified *M. oleifera* glucosinolates was investigated in three subsequent experiments using the Rhamno-Benzyl-GS and the Ac-Isomer-GS (Isomer

III) standards (individual glucosinolates). The total glucosinolate standard was also used in the 2nd and 3rd experiment. Stability tests were performed using buffered and/or unbuffered HPLC eluents. Experiments were replicated three times.

For the first experiment Rhamno-Benzyl-GS and Ac-Isomer-GS III were fractionated in unbuffered eluents, dried in a rotary evaporator (Heidolph VV 2000, bath WB 2000, vacuum pump: Vacuumbrand PC 3000 series, SVC3000), and re-dissolved in 4 ml water. 10 µl of the pure standards (Rhamno-Benzyl-GS > 95%, Ac-Isomer-GS III > 85%) were diluted in 1 ml water and checked for purity and stability immediately and after 9 days at room temperature or storage at -20 °C by HPLC analysis using unbuffered eluents.

In the second experiment buffered eluents were used for fractionation of Rhamno-Benzyl-GS and Ac-Isomer-GS III. Fractions were dried in a vacuum evaporator and re-dissolved in 4 ml water. 10 µl of the standards were diluted in 1 ml 0.1 M ammonium acetate buffer. The extract of the complete purified *Moringa* glucosinolates (total glucosinolate standard) was included in the second experiment. 10 µl of the total glucosinolate standard were diluted in 1 ml 0.1 M ammonium acetate buffer. Single glucosinolate standards and *M. oleifera* total glucosinolate standard were kept at room temperature, in a freezer at –20 °C, or thawed and refrozen three-times during the storage for 9 days. Standards were analyzed by HPLC using unbuffered and buffered eluents immediately and after 9 days.

The third experiment investigated the influence of buffer concentration on stability of compounds and conversion processes at room temperature. 10 µl of the total glucosinolate standard were diluted in different buffer concentrations (0 mM, 20 mM, 50 mM, 70 mM, 90 mM ammonium acetate) and glucosinolate profiles were analyzed at start and after 9 days by HPLC with buffered eluents.

**LC-MS analysis**

Identity of individual glucosinolates in samples of *M. oleifera* glucosinolates in desulfo and intact extracts were analyzed by LC-Ion Trap-MS system consisting of a 1100 series HPLC (Agilent Technologies, Germany) coupled to an Esquire 6000 ESI-Ion Trap mass spectrometer (Bruker Daltonics, Germany). Samples were separated on a 25 cm x 4.6 mm Nucleodur Sphinx RP column (5 µm, Macherey-Nagel, Germany) at 25 °C and 1 ml/min

flow using a binary gradient program with solvent A: 0.2% (v/v) formic acid, B: acetonitrile: 0 - 1 min: 1.5% B, 1 - 6 min: 1.5 - 5%, 6 - 8 min: 5 - 7% B, 8 - 18 min: 7 - 21% B, 18 - 23: 21 - 29%, 23 - 30 min: 29 - 43% B, 30 - 30.5 min: 43 - 100% B, 30.5 – 33 min: 100% B, 33 - 33.1 min: 100 - 1.5% B, and 33.1 - 35 min: 1.5% B. The eluent was split 1:3 delivering a flow of 250 µl/min to the ESI source, which was operated at the following parameters: skimmer voltage: 52.8 V; capillary exit voltage: 117 V; capillary voltage: -4,000 V/4000 V; nebulizer pressure: 35 psi; drying gas flow: 11 l/min; gas temperature: 330 °C alternating between +ve (desulfo glucosinolates) and –ve (intact glucosinolates) ion mode. MS spectra were recorded from m/z 60 – 1000. Retention times were 13.5 min for *p*-hydroxybenzyl glucosinolate and 14.7 min for Rhamno-Benzyl-GS for the analysis of desulfo glucosinolates and 11.6 min for Rhamno-Benzyl-GS, 17.0 min for Ac-Isomer-GS I, 17.8 min for Ac-Isomer-GS II, and 20.1 min for Ac-Isomer-GS III for the analysis for intact glucosinolates. Chromatograms of pure *Moringa* glucosinolates were recorded to relate identities between the chromatograms recorded on the Dionex HPLC system and the Merck Hitachi HPLC system.

**Statistical analysis**

Data were analyzed for significant differences using a one-way ANOVA followed by Tukey´s-HSD-test using the program SigmaPlot 12.0.

## Results

### Different HPLC profiles of *Moringa oleifera* were obtained using the desulfo and intact glucosinolate extraction method

HPLC and LC-MS analyses of desulfo and intact glucosinolates from *M. oleifera* revealed qualitative differences in the obtained profiles. The desulfo extraction method yielded only one major peak, Rhamno-Benzyl-GS, and one minor peak, eluting at the same time as *p*-hydroxybenzyl glucosinolate in the HPLC chromatogram (Figure 1a) whereas four glucosinolate peaks were identified in the chromatogram following the intact glucosinolate extraction method: Rhamno-Benzyl-GS and three Ac-Isomer-GSs (Figure 1b). Combined levels of Ac-Isomer-GSs nearly equaled the content of Rhamno-Benzyl-GS. *p*-Hydroxybenzyl glucosinolate was not detected in the intact glucosinolate

extract. *M. oleifera* desulfo glucosinolates were identified according to the mass spectra of protonated molecular ions $[M+H]^+$: (1) *p*-hydroxybenzyl glucosinolate *m/z* 346 (fragment at *m/z* 184) and (2) Rhamno-Benzyl-GS *m/z* 492 (fragments at *m/z* 330 and 184) (Figure 1c). The mass spectra of de-protonated molecular ions $[M-H]^-$ of intact glucosinolates were (1) Rhamno-Benzyl-GS (*m/z* 570) and (2) Ac-Isomer-GSs I - III (*m/z* 612) (Figure 1d).

*p*-Hydroxybenzyl glucosinolate represented an artifact of the desulfo glucosinolate extraction method deriving from Rhamno-Benzyl-GS through hydrolysis of rhamnose. This method also resulted in loss of acetyl-groups in Ac-Isomer-GSs leaving Rhamno-Benzyl-GS as the only detectable compound after desulfation with sulfatase. The chemical structure of the intact and desulfo glucosinolates, Rhamno-Benzyl-GS and Ac-Isomer-GS, in *M. oleifera* and possible breakdown/fragmentation products are shown in Figure 2.

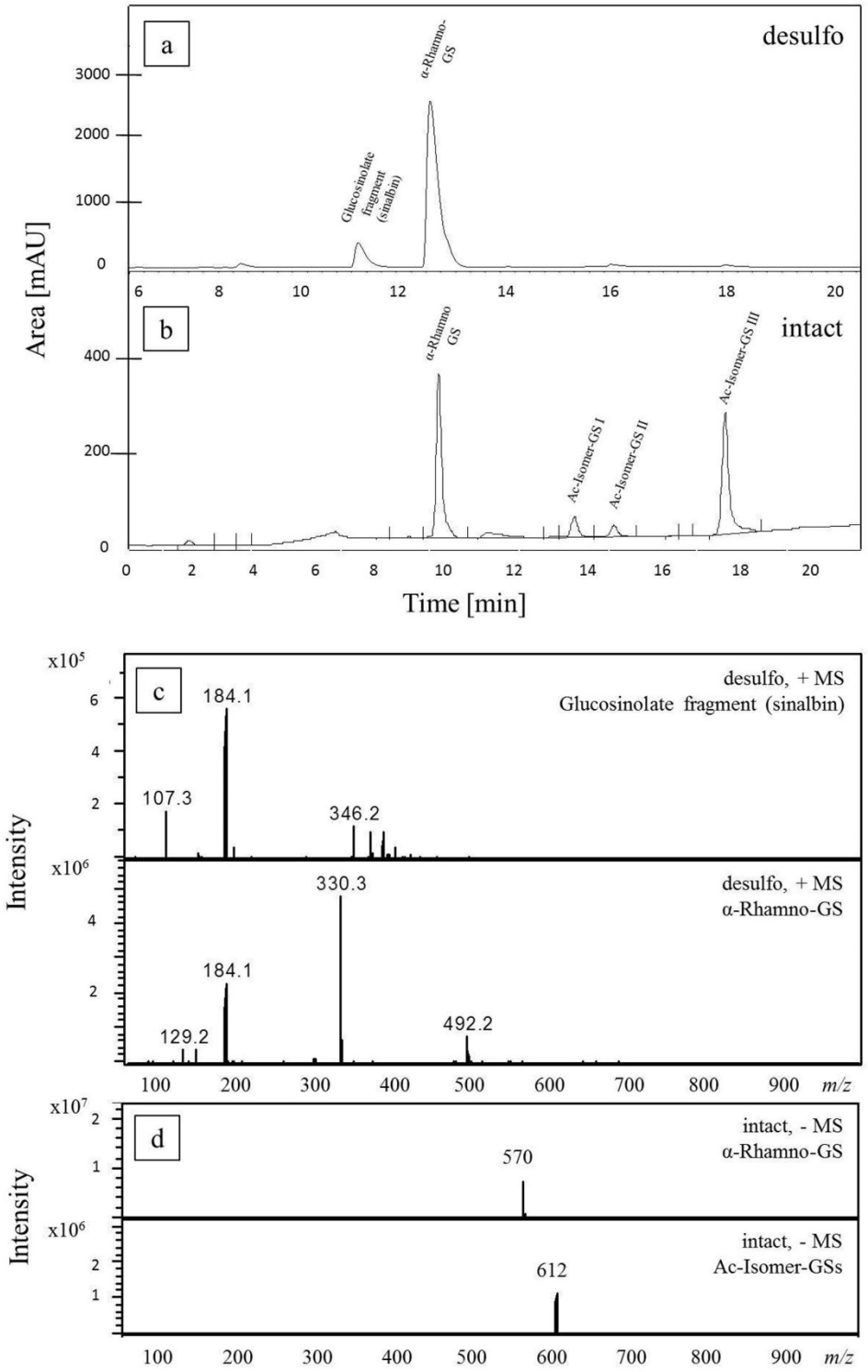



Fig. 1: Glucosinolate HPLC profiles obtained using the desulfo (a) and intact (b) glucosinolate extraction method und corresponding MS spectra obtained from desulfo (c) and intact glucosinolates (d)

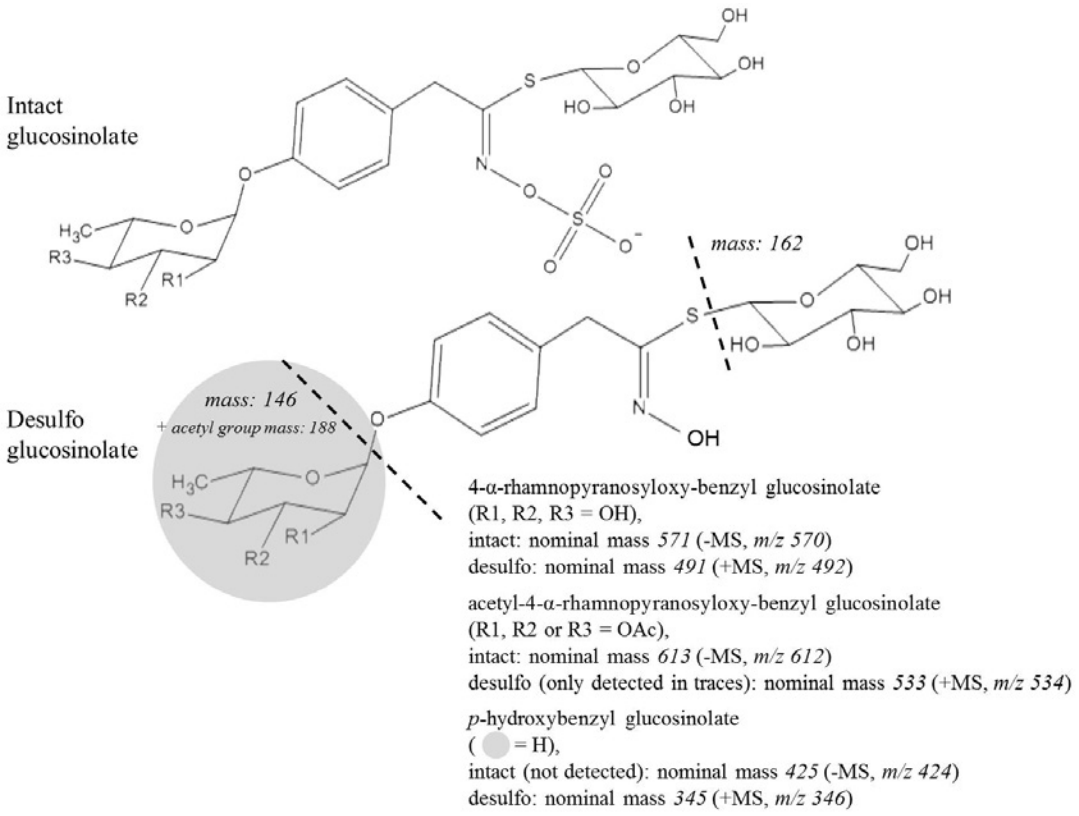



Fig. 2: Structure of the *Moringa oleifera* glucosinolate molecules formed by the intact or desulfo glucosinolate extraction method

In spite of the obvious qualitative differences in glucosinolate profiles between desulfo and intact glucosinolate extraction method, the resulting total glucosinolate amount did not differ significantly between the extraction methods for *M. oleifera* leaf material (Figure 3, one-way ANOVA, Tukey´s-HSD-test, $p < 0.05$). However, the precision of determination of glucosinolates based on the desulfo method was lower showing about twice the standard deviation than analyses based on the intact glucosinolate extraction method (Figure 3, $s = 4.5$, $s = 2.6$ respectively). Formation of artifact glucosinolates, loss of the acetylated glucosinolates, and lower precision of the desulfo glucosinolate extraction method showed that this method is not suitable for the analysis of glucosinolates in *M. oleifera* leaves.

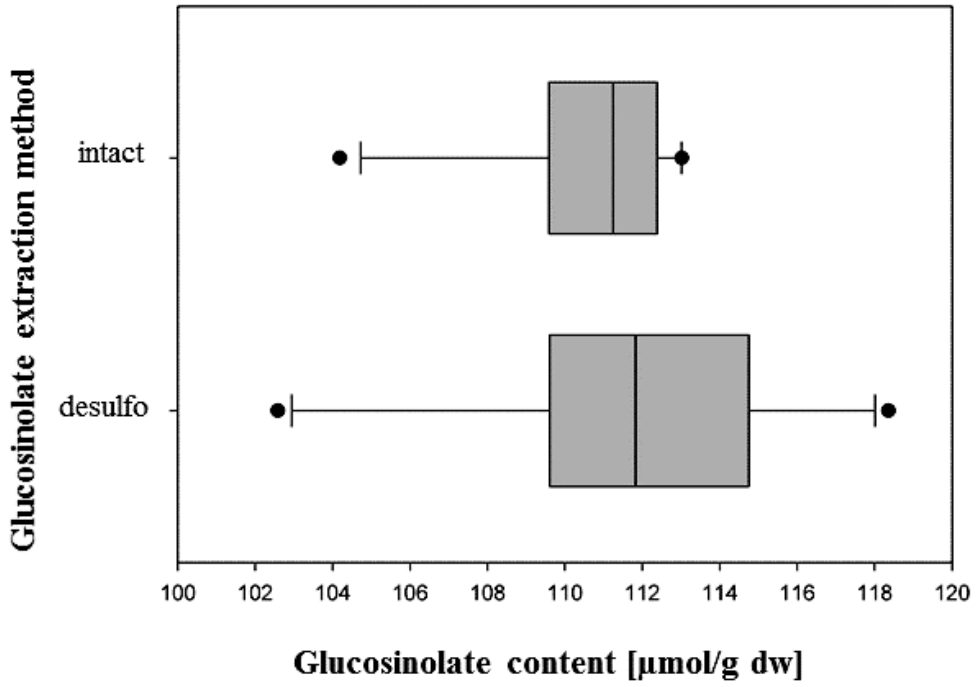



Fig. 3: Glucosinolate content of leaf material of one *Moringa oleifera* ecotypes analyzed with the desulfo and intact glucosinolate extraction method

**Stability of purified *Moringa oleifera* glucosinolates**

The results from desulfo method extracts indicated that glucosinolates of *M. oleifera* and specifically the three Ac-Isomer-GSs are liable to degradation and transformation processes. Therefore, stability of glucosinolates under different storage conditions and in buffered and unbuffered solvent systems was investigated in three consecutive steps.

*Degradation of glucosinolate standards in unbuffered conditions* (*1st stability test)*

Isolated and dried Rhamno-Benzyl-GS and Ac-Isomer-GS (Isomer III) standards (fractionated by HPLC with unbuffered eluents) were re-dissolved in water and stored at room temperature and at -20 °C. The glucosinolate profile of the Ac-Isomer-GS III was completely different after 9 days of storage in water at room temperature compared to storage at -20 °C while frozen fractions showed only little conversions or degradations of the Ac-Isomer-GS III (Suppl. Figure 2). At the beginning of the experiment, the Ac-Isomer-GS III represented 86% of the total glucosinolate amount (standard with 86% pureness, 3% Rhamno-Benzyl-GS, 8% Ac-Isomer-GS I, 3% Ac-Isomer-GS II). After 9

days at room temperature, only 29% on the total glucosinolate amount were contributed by the Ac-Isomer-GS III. While the proportion of Rhamno-Benzyl-GS increased only marginal from 3 to 6% of the total, the proportions of Ac-Isomer-GS Isomer I and Ac-Isomer-GS Isomer II increased to 40 and 25% of the total respectively. Interestingly, levels of Ac-Isomer-GS I and Ac-Isomer-GS II increased remarkably within the 9 days to levels equal or slightly higher than Ac-Isomer-GS III. Additionally, conversion processes were recognized after thawing and refreezing of the fractionated Ac-Isomer-GS III standard (data not shown). No conversion processes were found for the Rhamno-Benzyl-GS standard. Although it was possible to obtain a pure and stable standard of Rhamno-Benzyl-GS with our isolation and fractionation procedure in unbuffered conditions (unbuffered HPLC eluents, water as solvent), it was not possible to obtain a stable Ac-Isomer-GS III standard. The pure fractionated Ac-Isomer-GS III standard converted rapidly to a mixture of the three Ac-Isomer-GSs.

*Influence of unbuffered and buffered conditions on the stability of glucosinolate standards (2$^{nd}$ stability test)*

Because of the previous findings stability of Rhamno-Benzyl-GS, Ac-Isomer-GS III standards and also of total *Moringa* glucosinolate standard was investigated in water and buffered eluents (0.1 M ammonium acetate) under different storage conditions (room temperature, frozen, frozen with thawing). Total glucosinolate content of each standard degraded significantly when stored at room temperature for 9 days, falling to 11.26 - 39.71% of initial levels depending on the standard and HPLC eluents used (Table 1). Degradation was significantly lower in frozen samples ranging from 2.22 to 11.26% and similarly, in samples, which had been thawed and refrozen repeatedly. Degradation of the total glucosinolate content in each standard stored at room temperature was significantly lower when analyses were carried out in buffered HPLC conditions (total glucosinolate standard $p < 0.001$, Rhamno-Benzyl-GS $p = 0.011$, Ac-Isomer-GS III $p = 0.002$). Glucosinolate composition in standards of total glucosinolate and Ac-Isomer-GS III also changed rapidly (Figure 4) in addition to reduction of total glucosinolate content of the different standards (Table 1). The proportion of Ac-Isomer-GS III fell from 81.1% to 21.84% of the total after 9 days at room temperature and under buffered conditions, while proportions of Ac-Isomer-GS I, Ac-Isomer-GS II and Rhamno-Benzyl-

GS increased correspondingly (Figure 4). A similar change in isomer proportions was also observed for the total glucosinolate standard.

Tab. 1: Degradation of the total glucosinolate content of in buffer dissolved purified glucosinolates related to initial determined amounts (100%) under different storage conditions measured using buffered or unbuffered eluents after 9 days (one-way ANOVA, Tukey´s-HSD-test, $p < 0.04$, initial determined amounts were determined as "a" for statistical analysis)

| | | **Percentage [%] of remaining GS of initial amounts after 9 days** | | |
|---|---|---|---|---|
| | | **Storage condition** | | |
| **Purified GS** | **HPLC eluents** | **Room temperature** | **Frozen** | **Frozen incl. thawing** |
| **Total isolated GSs** | **Unbuffered** | 67.72 c | 94.11 ab | 84.36 b |
| | **Buffered** | 88.74 b | 96.35 ab | 89.01 ab |
| **Rhamno-Benzyl-GS** | **Unbuffered** | 60.29 b | 96.90 a | 99.48 a |
| | **Buffered** | 73.55 d | 88.74 c | 94.38 b |
| **Ac-Isomer-GS III** | **Unbuffered** | 65.52 b | 97.73 a | 96.74 a |
| | **Buffered** | 79.05 b | 91.41 ab | 90.59 ab |

GS = glucosinolate

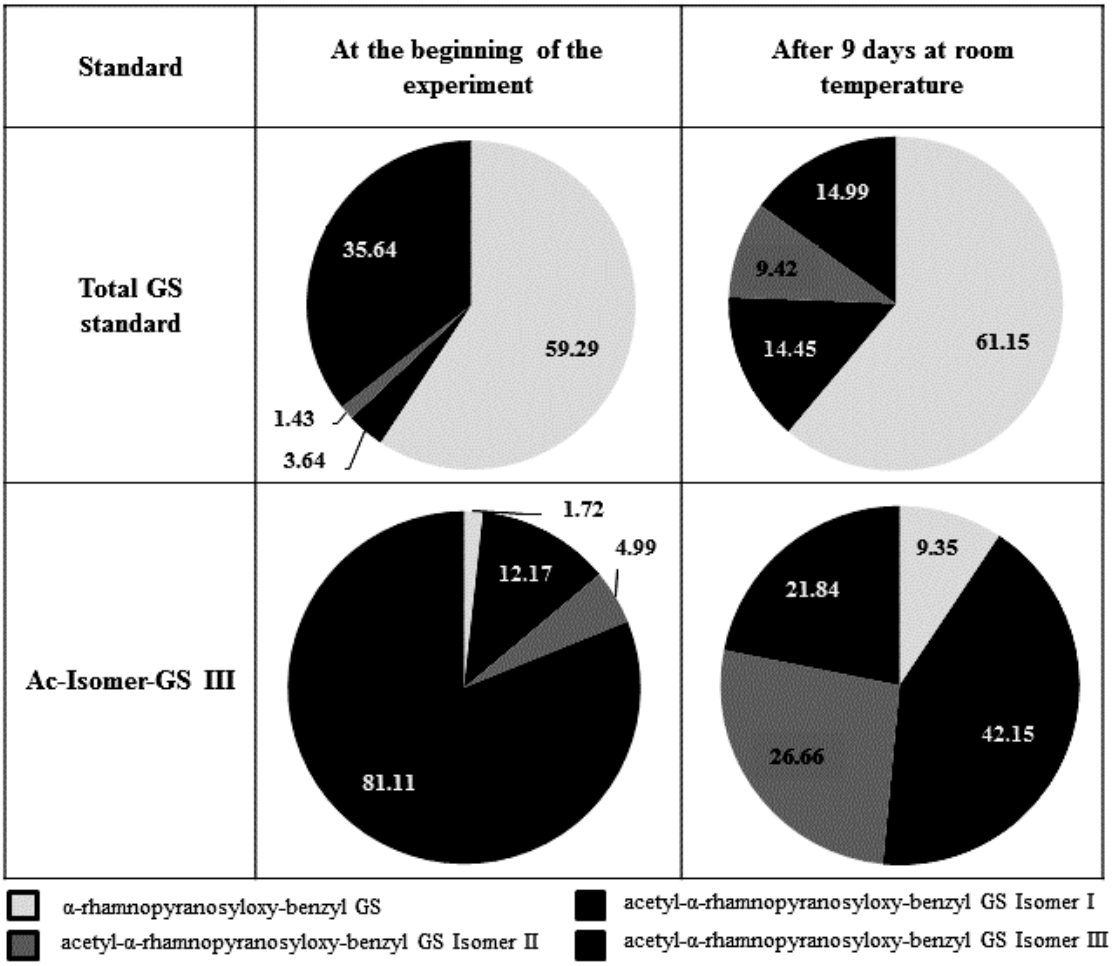



Fig. 4: Percentage of single glucosinolates on total glucosinolate amounts in the total isolated glucosinolate standard and the acetyl-4-α-rhamnopyranosyloxy-benzyl glucosinolate standard (Isomer III) determined initially and after 9 days in buffered conditions at room temperature

*Glucosinolate conversion processes of standards dissolved in different buffer concentrations (3rd stability test)*

The conversion of the Ac-Isomer-GS III into different Ac-Isomer-GSs as described before was also observed for the total glucosinolate standard and increased with increasing buffer concentration (Table 2). As expected the proportion of the Ac-Isomer-GS III decreased, while proportions of Ac-Isomer-GS I and II increased with increasing buffer concentrations (0 mM, 20 mM, 50 mM, 70 mM, 90 mM ammonium acetate).

However, the total degree of degradation of the glucosinolates was reduced with the increasing buffer concentration.

Tab. 2: Glucosinolate degradation and conversion processes of the total isolated glucosinolate standard in different buffer concentrations after 9 days at room temperature, **a)** individual glucosinolate percentage on the total isolated glucosinolate standard [%], **b)** change of the glucosinolate content of single glucosinolates [%]

| | **Rhamno -Benzyl- GS** | **Ac- Isomer- GS I** | **Ac- Isomer- GS II** | **Ac- Isomer -GS III** | **Total** |
|---|---|---|---|---|---|
| | | | **a** | | |
| | **Beginning of the experiment** | | | | |
| **Buffer concentration [mM]** | 61.11 | 2.31 | 1.13 | 35.46 | 100 |
| | **After 9 days** | | | | |
| **0** | 61.95 | 2.98 | 1.20 | 33.86 | 100 |
| **20** | 61.35 | 6.09 | 2.74 | 29.82 | 100 |
| **50** | 60.73 | 9.23 | 4.97 | 25.06 | 100 |
| **70** | 61.54 | 11.02 | 6.34 | 21.10 | 100 |
| **90** | 61.22 | 12.99 | 7.20 | 19.29 | 100 |
| | | | **b** | | |
| | **Beginning of the experiment** | | | | |
| **Buffer concentration [mM]** | 100 | 100 | 100 | 100 | **100** |
| | **After 9 days** | | | | |
| **0** | -39.34 | -22.71 | -36.12 | -42.86 | **-40.17** |
| **20** | -41.38 | +40.40 | +35.96 | -51.65 | **-42.09** |
| **50** | -32.31 | +108.29 | +167.52 | -54.82 | **-33.99** |
| **70** | -35.55 | +110.05 | +197.85 | -62.41 | **-37.10** |
| **90** | -32.26 | +122.33 | +229.36 | -64.06 | **-34.11** |

GS = glucosinolate

## Discussion

In the present paper, we investigated two existing glucosinolate extraction methods with the aim to determine a method, which allow a reliably determination of glucosinolate profiles of *M. oleifera* leaves. Chromatograms of glucosinolate extracts of leaf material using the desulfo and intact glucosinolate extraction method revealed completely different glucosinolate profiles. Rhamno-Benzyl-GS and three Ac-Isomer-GSs were detected with HPLC, with the Ac-Isomer-GS Isomer III as the second most abundant compound next to Rhamno-Benzyl-GS when using the intact extraction method. By contrast, only Rhamno-Benzyl-GS and an artifact, *p*-hydroxybenzyl glucosinolate, which is formed from Rhamno-Benzyl-GS through hydrolytic cleavage of rhamnose, were found in extracts using the desulfo glucosinolate extraction method. The total loss of acetyl isomers in extracts following the desulfo protocol suggests the presence of hydrolytic conditions during the extraction.

Hydrolysis of acetyl groups and sugars is performed easily and widely used in natural product chemistry. For example, McMurrough et al. (1982) used acid hydrolysis to determine the natures of aglycons and sugars of flavonol glycosides of hops. Acid hydrolysis of naturally occurring flavonoids is often used to release the sugar residues linked to the flavonoid aglycon enabling a further study of the flavonoids aglycon (Lee et al., 1995). Using this method Sharaf et al. (1997) identified several flavonoids in *Cleome* and *Capparis* species by determining different sugars (rhamnose, glucose) in the flavonoid molecule. Thomas et al. reported already 1958 that an acid hydrolysis of naringin resulted in the degradation products rhamnose, glucose and the aglycon, naringenin.

The elution of methanolic extracts of glucosinolates from DEAE Sephadex with $K_2SO_4$ yielded all Ac-Isomer-GSs in the intact glucosinolate extract from *M. oleifera* (see section: 2.3.1) although preparation of DEAE Sephadex anion exchanger columns was the same as for the desulfo glucosinolate extraction method. Thies (1988) reported that this step in the intact glucosinolate extraction method is in principle suitable for preparation of all intact glucosinolates. The only differences in column conditions after application of methanolic extracts between the intact glucosinolate extraction procedure

and the desulfo glucosinolate extraction method were application of sodium acetate buffer (pH = 4), of sulfatase solution, and incubation of glucosinolates on the column at pH 4 and room temperature for about 12 h. It is reasonable to assume that overnight incubation (nearly 12 h) of glucosinolate extracts with sulfatase at room temperature and at pH 4 may facilitate the observed hydrolysis of the glucosinolates. Acidic hydrolysis depends on temperature, time, and acid concentration in general (Jeevan et al., 2011) and Varki & Diaz (1984) reported losses of 30 - 60% of acetyl groups from of sialic acids through ion-exchange chromatography. Hydrolysis would favour cleavage of the acetyl groups but also lyse the more stable glycosidic bond of the rhamnose moiety from the glucosinolate could be the result (Figure 2), leading *p*-hydroxybenzyl glucosinolate, which was observed as artifact and does not occur naturally in *M. oleifera* leaves.

In contrast to the complete change of the *M. oleifera* glucosinolate profile when using the two different glucosinolate extraction methods, the quantitation of total glucosinolate content was nearly the same with both extraction methods but did show a higher variance for total glucosinolates from desulfo extracts. Appearance of artifacts was also reported by other authors using the standard desulfo extraction method. For example, Bennett et al. (2007) observed artifacts formed during extraction of 4-mercaptobutyl glucosinolate, the major glucosinolate of *Eruca sativa*, which oxidized forming two a dimer and two disulfide derivatives.

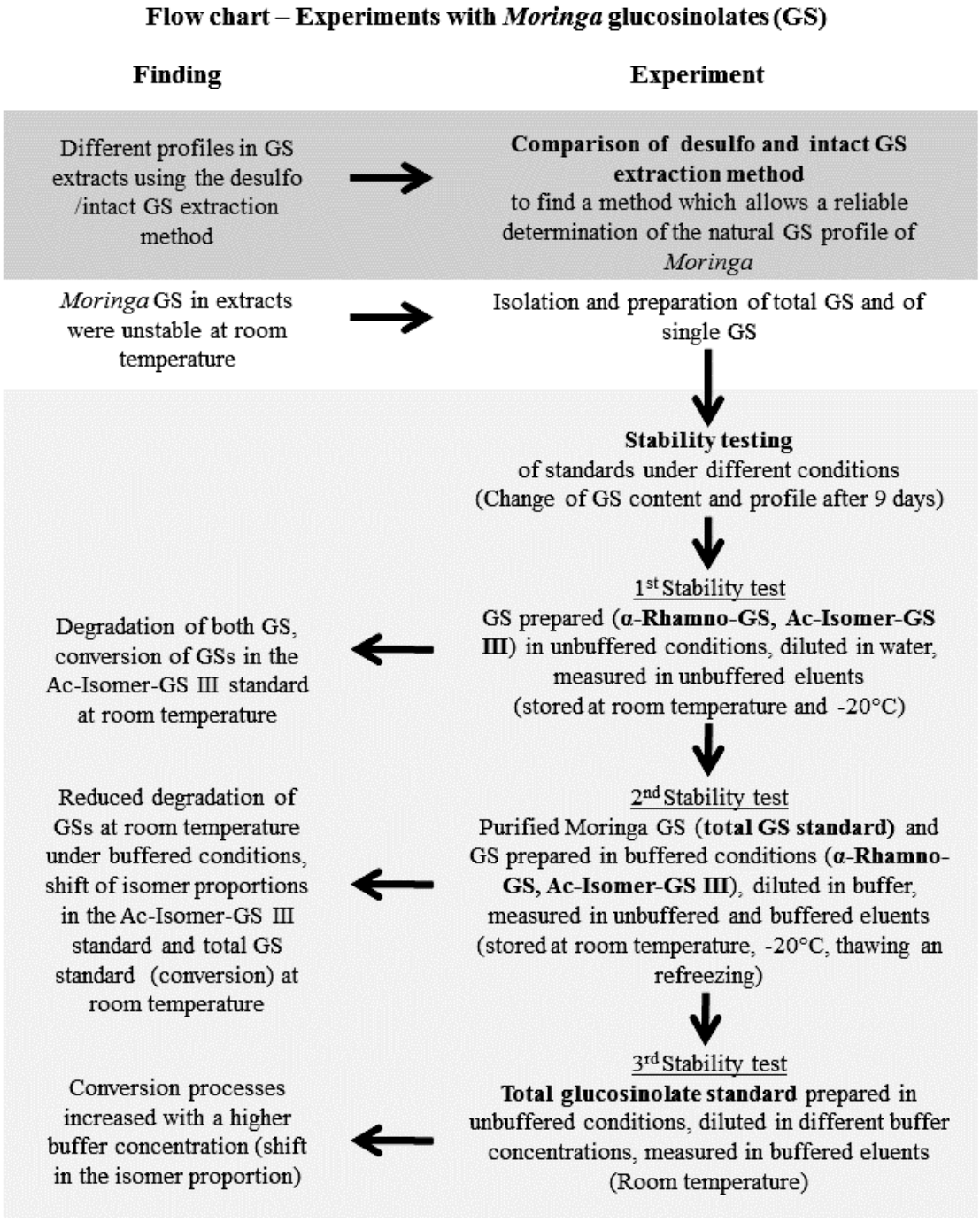
The acetyl isomers of Rhamno-Benzyl-GS were not only sensitive to the extraction method used but also showed considerable re-arrangement activity at room temperature and in buffered solution. For example standards of Ac-Isomer-GS III converted over time to Ac-Isomer-GSs I and II and Rhamno-Benzyl-GS yielding approximately equal amounts of all three Ac-Isomer-GSs after 9 days. Only slight conversion processes were detected after three freeze-thaw cycles of Ac-Isomer-GS. Apparently, the acetyl group was able to move between the three hydroxyl groups of the rhamnopyranose. A similar phenomenon was reported by Varki & Diaz (1984) for acetylated sialic acids who found that re-arrangement of acetyl groups on sialic acids was affected by temperature and pH. Roslund et al. (2008) demonstrated the migration of acetyl groups on galactopyranos and were able to minimize these conversion processes during HPLC by using ammonium acetate buffer as eluents. Our study confirmed these findings for *Moringa* glucosinolates

as single and total standards maintained their integrity during HPLC analyses when using 0.1 M ammonium acetate buffer.

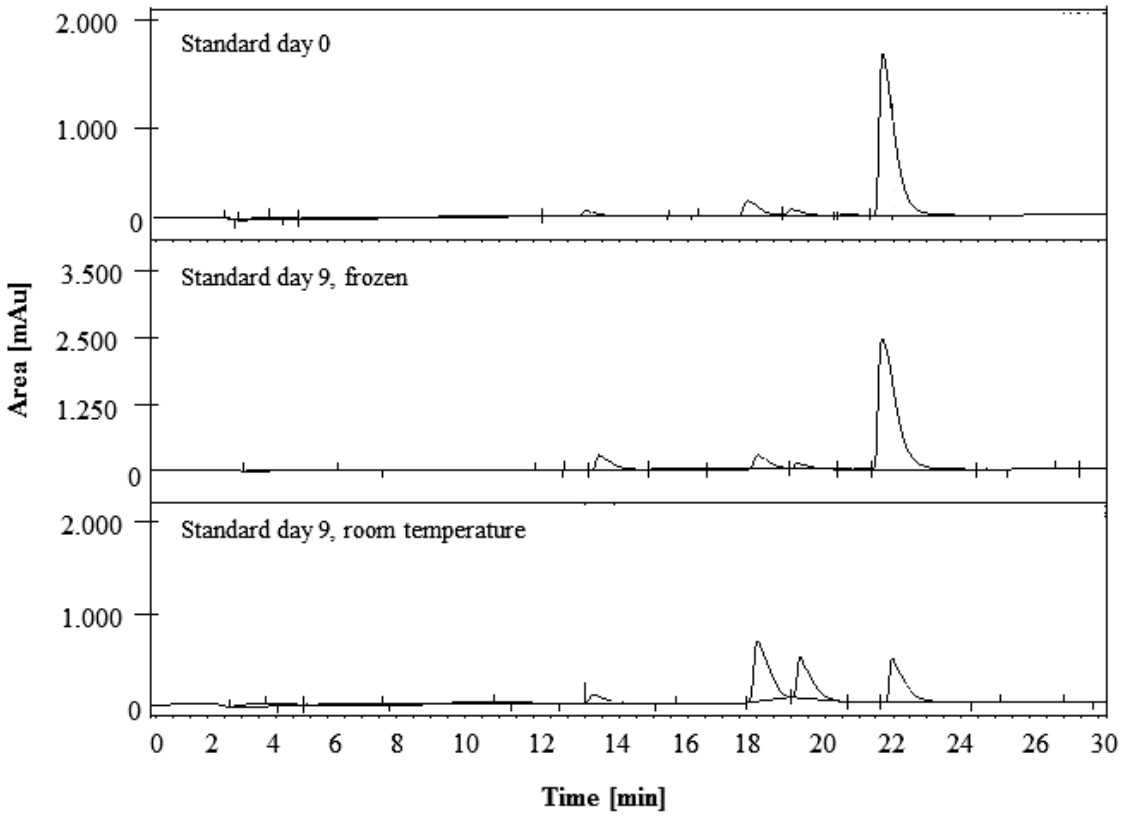
A very slow loading of solutions and methanolic glucosinolate extract onto the anion exchanger increased total glucosinolate yield of our glucosinolate purification method (results not shown), which can be explained by the polarity of the glucosinolates. Linkage to a sugar molecule increases the polarity of the molecule and consequently the water solubility. Aherne & O'Brien (2002) reported this finding for flavonols, where a glycosidic linkage increased the flavonol polarity. Different glucosinolates vary in their affinity to DEAE Sephadex anion exchanger A25. 2-Propenyl glucosinolate and benzyl glucosinolate have a very low affinity to DEAE Sephadex, indole glucosinolates and glucosinolates substituted with phenolic acyl groups show a high affinity (Agerbirk & Olsen, 2012). The presence of a rhamnose and additional acetyl group in the molecules of *Moringa* glucosinolates might result in a lower glucosinolate affinity to DEAE Sephadex. In any case, the elution volume of glucosinolates on DEAE Sephadex depends on the individual glucosinolate and has not yet been analyzed sufficiently for acylated glucosinolates (Agerbirk & Olsen, 2012). The glucosinolate content of leaf material of *M. oleifera* is also very high compared to Brassicaceae (up to 600 µmol/g dry weight) and fast application of extracts in combination with a comparably short column and potentially extended time to achieve on exchange equilibrium may essentially overload the column.

Our results showed that the desulfo glucosinolate extraction method resulted in the production of artifact glucosinolates and a loss of acetylated glucosinolates and that it is, therefore, not adequate for analysis of individual *M. oleifera* glucosinolates. The optimized intact extraction method described here was able to determine reliably the natural glucosinolate profile in *M. oleifera* leaves. Because of rapid degradation of standards at room temperature, frozen storage is recommended, which is not affected by repeated freeze-thaw cycles.

## Supplemented Figures




Suppl. Fig. 1: Flow chart of consecutive performed experiments (test sequence)




Suppl. Fig. 1: Chromatograms of the acetyl-4-α-rhamnopyranosyloxy-benzyl glucosinolate (Isomer III) standard stored frozen or at room temperature for 9 days

## References

Agerbirk, N. & Olsen, C. E. (2012): Glucosinolate structures in evolution. *Phytochemistry* 77: 16-45.

Aherne, S. A. & O'Brien, N. M. (2002): Dietary flavonols: chemistry, food content and metabolism. *Nutrition* 18: 75-81.

Bennett, R. N.; Carvalho, R.; Mellon, F. A.; Eagles, J. & Rosa, E. A. S. (2007): Identification and quantification of glucosinolates in sprouts derived from seeds of wild *Eruca sativa* L. (salad rocket) and *Diplotaxis tenuifolia* L. (wild rocket) from diverse geographical locations. *Journal of Agricultural and Food Chemistry* 55: 67-74.

Bennett, R. N.; Mellon, F. A. & Kroon, P. A. (2004): Screening crucifer seeds as source of specific intact glucosinolates using ion-pair high-performance liquid chromatography negative ion electrospray mass spectrometry. *Journal of Agricultural and Food Chemistry* 52: 428-438.

Bennett, R. N.; Mellon, F. A.; Foidl, N.; Pratt, J. H.; Dupont, M. S.; Perkins, L. & Kroon, P. A. (2003): Profiling glucosinolates and phenolics in vegetative and reproductive tissues of the multi-purpose trees *Moringa oleifera* L. (Horseradish Tree) and *Moringa stenopetala* L. *Journal of Agricultural and Food Chemistry* 51: 3546-3553.

Cáceres, A.; Cabrera, O.; Morales, O.; Millinedo, P.; Mendia, P. (1991): Pharmacologic properties of *Moringa oleifera*. 1: Preliminary screening for antimicrobial activity. *Journal of Ethnopharmacology* 33: 213-216.

Cáceres, A.; Saravia, A.; Rizzo, S.; Zabala, L.; De Leon, E. & Nave, F. (1992): Pharmacologic properties of *Moringa oleifera*. 2: Screening for antispasmodic, anti-inflammatory and diuretic activity. *Journal of Ethnopharmacology* 36: 233-237.

Clarke, D. B. (2010): Glucosinolates, structures and analysis in food. *Analytical Methods* 2: 310-325.

Dam, N. M. van, Tytgat, T. O. G. & Kirkegaard, J. A. (2009): Root and shoot glucosinolates: A comparison of their diversity, function and interactions in natural and managed ecosystems. *Phytochemistry Reviews* 8: 171-186.

European Economic Community (EEC): *Commission Regulation (EEC) No 2435/86* of 29 July 1986 amending Regulation (EEC) No 1470/68 on the drawing and reduction of samples and the determination of the oil content, impurities and moisture in soil seeds.

Fahey, J. W.; Zalcmann, A. T. & Talalay, P. (2001): Chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56: 5-51.

Guevara, A. P.; Vargas, C.; Sakurai, H.; Fujiwara, Y.; Hashimoto, K.; Maoka, T.; Kozuka, M.; Ito, Y.; Tokuda, H. & Nishino, H. (1999): An antitumor promotor from *Moringa oleifera* Lam. *Mutation Research* 440: 181-188.

Iori, R.; Barillari, J.; Gallienne, E.; Bilardo, C.; Tatibouët, A. & Rollin, P. (2008): Thio-functionalised glucosinolates: unexpected transformation of desulfoglucoraphenin, *Tetrahedron Letters* 49: 292-295.

Jeevan, P., Nelson, R. & Rena, A. E. (2011): Optimization studies on acid hydrolysis of Corn cob hemicellulosic hydrolysate for microbial production of xylitol. *Journal of Microbiology and Biotechnology Research* 1: 114-123.

Lee, Y., Howard, L. R. & Villalón, B. (1995): Flavonoid and antioxidant activity of fresh pepper (*Capsicum annuum*) cultivars. *Journal of Food Science* 60: 473-476.

Leur, H. van, Raaijmakers, C. E. & Dam, N. M. van (2006): A heritable glucosinolate polymorphism within natural populations of *Barbarea vulgaris*. *Phytochemistry* 67: 1214-1223.

Leur, H. van, Vet, L. E. M., van der Putten, W. H. & Dam, N. M. van (2008): *Barbarea vulgaris* glucosinolate phenotypes differentially affect performance and preference of two different species of lepidopteran herbivores. *Journal of Chemical Ecology* 34: 121-131.

Lewke, A., Hanke, A., Schnitzler & W. H. (1996): HPLC-Analytik von intakten Glucosinolaten in Gemüsebrassicaceae und deren enzymatischer Nachweis durch Myrosinaseabbau. *Gartenbauwissenschaft* 61: 179-183.

McMurrough, I., Hennigan, G. P. & Loughrey, M. J. (1982): Quantitative analysis of hop flavonols using high performance liquid chromatography. *Journal of Agricultural and Food Chemistry* 30: 1102-1106.

Melchini, A., Traka, M. H., Catania, S., Miceli, N., Taviano, M. F., Maimone, P., Francisco, M., Mithen, R. F. & Costa, C. (2013): Antiproliferative activity of the dietary isothiocyanate erucin, a bioactive compound from cruciferous vegetables, on human prostate cancer cells. *Nutrition and Cancer* 65: 132-138.

Mewis, I., Appel, H. M., Hom, A., Raina, R. & Schultz, J. C. (2005): Major signaling pathways modulate Arabidopsis glucosinolate accumulation and response to both phloem-feeding and chewing insects. *Plant Physiology* 138: 1149-1162.

Mewis, I., Khan, M. A. M., Glawischnig, E., Schreiner, M. & Ulrichs, C. (2012): Water stress and aphid feeding differentially influence metabolite composition in Arabidopsis thaliana (L.). *PLoS ONE* 7, e48661, doi:10.1371/journal.pone.0048661.

NA 057-05-05 AA - Joint committee of DIN and DGF for the analysis of fats, oils and products thereof, related and primary products. (2012): Rapeseed - Determination of glucosinolate content - Part 1: Method using high-performance liquid chromatography (*ISO 9167-1:1992/DAM 1:2012*), German version EN ISO 9167-1:1995/prA1:2012.

Prakash, D. & Gupta, C. (2012): Glucosinolates: The phytochemicals of nutraceutical importance. *Journal of Complementary and Integrative Medicine* 9, doi: 10.1515/1553-3840.1611.

Roslund, M. U., Aitio, O., Wärnå, J., Maaheimo, H., Murzin, D. Y. & Leino, R. (2008): Acyl group migration and cleavage in selectively protected β-d-galactopyranosides as studied by NMR spectroscopy and kinetic calculations. *Journal of the American Chemical Society* 130: 8769-8772.

Sharaf, M., El-Ansari, M. A. & Saleh, N. A. M. (1997): Flavonoids of four *Cleome* and three *Capparis* species. *Biochemical Systematics and Ecology* 25: 161-166.

Singh, K. K., Kumar, K. (1999): Ethnotherapeutics of some medicinal plants used as antipyretic agents among the tribals of India. *Journal of Economic and Taxonomic Botany* 23: 135-141.

Thies, W. (1988): Isolation of sinigrin and glucotropaeolin from cruciferous seeds. *European Journal of Lipid Science and Technology* 8: 311-314.

Thomas, D. W., Smythe, C. V. & Labbee, M. D. (1958): Enzymatic hydrolysis of naringin, the bitter principle of grapefruit. *Journal of Food Science* 23: 591-598.

Varki, A. & Diaz, S. (1984): The release and purification of sialic acids from glycoconjugates: Methods to minimize the loss and migration of *O*-acetyl groups. *Analytical Biochemistry* 137: 236-247.

Verkerk, R., Schreiner, M., Krumbein, A., Ciska, E., Holst, B., Rowland, I., Schrijver, R. de, Hansen, M., Gerhäuser, C., Mithen, R.& Dekker, M. (2009): Glucosinolates in

Brassica vegetables: The influence of the food supply chain on intake, bioavailability and human health. *Molecular Nutrition & Food Research* 53: 219-265.

## Kapitel IV - Antioxidatives und mutagenes Potential von *M. oleifera*-Glucosinolaten

Wie bereits ausgeführt, wird Hydrolyseprodukten von einigen Glucosinolaten ein antioxidatives sowie antikarzinogenes Potential zugeschrieben (u. a. reviewed in Clarke et al., 2008; Zhang & Talalay, 1998). Wie u. a. für Sulforaphan oder Phenylethyl-Isothiocyanat gezeigt werden konnte (Krajka-Kuzniak et al., 2015; Saw et al., 2011), können Glucosinolate die Expression von Genen, welche Phase II- sowie antioxidative Enzyme kodieren, beeinflussen. Diese Stimulation erfolgt über den Nrf2/ARE-Signalweg (Haack et al., 2010; Krajka-Kuzniak et al., 2015; Saw et al., 2011). Dieses wird als ein Indiz auf ein antioxidatives sowie antikarzinogenes Potential des Glucosinolates gewertet. Im Gegensatz dazu wurden in Studien jedoch auch mutagene und bakteriotoxische Effekte von Glucosinolaten nachgewiesen (Baasanjav-Gerber et al., 2011; Glatt et al., 2011).

*M. oleifera* weist sehr hohe Gehalte von strukturell selten vorkommenden Glucosinolaten auf (Bennett et al., 2003; Förster et al., 2015a). Hierbei ist am Aglykon des aromatischen Glucosinolates (Phenolring) ein weiteres Zuckermolekül, die Rhamnose, gebunden. Untersuchungen von Cham (2007) zeigten, dass durch ein Zuckermolekül besondere Molekülstrukturen entstehen können, welche antikarzinogene Effekte begründen können. So fanden Cham & Daunter (1990) heraus, dass die Bindung von Solamargin an Tumorzellen durch die Rhamnose vermittelt wurde. Für die Glucosinolate von *M. oleifera* sollten daher folgende Aspekte untersucht werden:

- *Sind zytotoxische Effekte von M. oleifera-Glucosinolaten an HepG2-Zellen nachzuweisen?*
- *Besitzen die Glucosinolate von M. oleifera das Potential antioxidativ und antikarzinogen zu wirken?*
- *Werden die Effekte über den Nrf2/ARE-Signalweg vermittelt?*
- *Wird die Transkription und Translation von Nrf2-Zielgenen durch die Glucosinolate von M. oleifera in HepG2-Zellen beeinflusst?*
- *Können für die M. oleifera-Glucosinolate mutagene und genotoxische Effekte nachgewiesen werden?*

Die in diesem Kapitel dargestellten Fragenstellungen werden in folgender Publikation behandelt:

> Förster, N.; Mewis, I.; Glatt, H. R.; Haack, M.; Brigelius-Flohé, R.; Schreiner, M. & Ulrichs, C. (2016): Characteristic single glucosinolates from *Moringa oleifera*: Induction of detoxifying enzymes and lack of genotoxic activity in various model systems. *Food & Function* 7: 4660-4674.

## Abstract

Leaves of *Moringa oleifera* are used in tribes as biological cancer medicine. Scientific investigations with *M. oleifera* conducted so far used almost exclusively total plant extracts. Studies on the activity of single compounds are missing. Therefore, biological effects of the two main aromatic multi-glycosylated glucosinolates of *M. oleifera* were investigated in the present study. The cytotoxic effects of the *M. oleifera* glucosinolates were identified for HepG2 cells (NRU assay), for V79-MZ cells (HPRT assay, SCE assay), and for two *Salmonella typhimurium* strains (Ames test). Genotoxic effects of these glucosinolates were not observed (Ames test, HPRT assay, and SCE assay). Reporter gene assays revealed a significant increase in the ARE-dependent promoter activity of *NQO1* and *GPx2* indicating an activation of the Nrf2 pathway by *M. oleifera* glucosinolates. Since both enzymes can also be induced via activation of the AhR, plasmids containing promoters of both enzymes mutated in the respective binding sites (pGL3enh-h*NQO1*-ARE, pGL3enh-h*NQO1*-XRE, pGL3bas-h*GPX2*-mutARE, pGL3bas-h*GPX2*-mutXRE) were transfected. Analyses revealed that the majority of the stimulating effects was mediated by the ARE motif, whereas the XRE motif played only a minor role. The stimulating effects of *M. oleifera* glucosinolates could be demonstrated both at the transcriptional (reporter gene assay, real time-PCR) and translational level (enzyme activity) making them interesting compounds for further investigation.

## Introduction

Cancer is still the most frequent cause of human death all over the world (8.2 million cancer-related deaths in 2012; Steward & Wild, 2014). Next to chemo- and radiotherapy in oncology as common methods to fight cancer, medical plants, used as herbal drugs in traditional and tribal medicine for centuries, are still objects of research. Several authors reported that herbs and herbal extracts possess cancer-preventing properties (e. g. Aruna & Sivaramakrishnan, 1992; reviewed in Craig, 1999), e. g. *Allium* sp. (garlic, onion), members of the Labiatae family (basil, mints, thyme), Zingiberaceae family (turmeric, ginger), or Umbelliferae family (anise, caraway, coriander, cumin). According to literature, these antioxidative, anticarcinogenic, and anti-inflammatory effects depend on

plant secondary metabolites such as flavonoids, alkaloids, phenolic acids, and glucosinolates.

Because of its high nutritional value, *Moringa oleifera* – the so-called miracle tree – is used to overcome famine and malnutrition, especially in Africa. Leaves, bark, and seeds were used in form of extracts, teas, or ointments to fight eye-, ear- and skin-infections, fever, swelling, headaches, intestinal worms (reviewed in Mahmood & Ul Haq, 2010) and various tumors (Hartwell, 1971). The anticarcinogenic potential of aqueous, ethanolic, and methanolic extracts of total leaf, seed, pod or root bark as well as plant material and individual substances of *M. oleifera* has been demonstrated in *in vitro* and *in vivo* studies. Underlying mechanisms include the modulation of phase I, phase II, and antioxidant enzymes thereby suppressing, for example, skin or colon papillomagenesis in cell systems and mice (Bharali et al., 2003; Budda et al, 2011; Rahan et al., 2016; Sharma & Paliwal, 2014). Furthermore, cytotoxic effects of *M. oleifera* extracts on different cancer cell lines were described (Costa-Lotufo et al., 2005; Khalafalla et al., 2010; Parvathy & Umamaheshwari, 2007). Particularly the leaves of *M. oleifera* are characterized by very high contents of multi-glycosylated aromatic glucosinolates (Bennett et al., 2003; Förster et al., 2015a). Other glucosinolates, in particular their metabolites, isothiocyanates, are known to influence the expression of detoxifying enzymes, too. For example sulforaphane, a breakdown product of glucoraphanin (4-methylsulfinylbutyl glucosinolate) present, for example, in broccoli, induces phase I and phase II enzymes and/or initiates apoptosis and cell cycle arrest (Clarke et al., 2008; Fahey et al., 1997; Zhang et al., 1992; Zhang & Talalay, 1998). Isothiocyanates (-N=C=S), glucosinolate hydrolysis products, act as Michael reaction acceptors (Talalay, 2000) and therefore play a major role in the induction of phase II enzymes and enzymes of the defense system via Nrf2 (Krajka-Kuzniak et al., 2015). Next to sulforaphane, phenethyl isothiocyanate was shown to have similar effects (Krajka-Kuzniak et al., 2015; Krajka-Kuzniak et al., 2011; Saw et al., 2011).

*M. oleifera* is often used in tribal medicine but scientific investigations conducted so far focused almost exclusively on total plant extracts. Therefore, we investigated the biological effects of the characteristic aromatic multi-glycosylated glucosinolates found in *M. oleifera*. The performed assays were carried out with the two main glucosinolates

from leaf extracts of *M. oleifera*: the 4-α-rhamnopyranosyloxy-benzyl glucosinolate (Rhamno-Benzyl-GS) and acetyl-4-α-rhamnopyranosyloxy-benzyl glucosinolate Isomer III (Ac-Isomer-GS III) (structural formulas in Suppl. Figure 2). The aim of this study was to identify putative cytotoxic effects of hydrolyzed glucosinolates on HepG2 cells as well as the potential to protect against inflammatory and carcinogenic stimuli. Additionally, it was analyzed whether the *M. oleifera* glucosinolates stimulate phase II and antioxidant enzymes via the Nrf2/ARE and/or the AhR/XRE pathways. Stimulation of genes encoding for enzymes associated with cancer-inhibiting or prevention potential was analyzed in the luciferase reporter gene assays. Genes encoding NQO1, GPx2, and Srx were selected. These enzymes belong to those of the defense system which detoxify and eliminate xenobiotics and reactive metabolites, including $H_2O_2$ and other reactive oxygen species (ROS) that may cause damage to cells and tissues (Matés et al., 1999; Yang et al., 2010). These reactive molecules may increase risk of cancer and other chronic degenerative diseases probably associated with oxidative stress (Kwak et al., 2004; Talalay et al., 2003; Yu & Hensler, 2005). Various genes that encode phase II and enzymes of the defense system contain an ARE motif in their promoter region (Nguyen et al., 2003) and are therefore induced via the Nrf2 pathway. *NQO1* (Nioi & Hayes, 2004), *GPx2* (Banning et al., 2005), Srxn1 (Kim et al., 2010), and *TrxR1* (Hintze et al., 2003) belong to the group of Nrf2 target genes. As reviewed in Denison et al. (2002), also different secondary plant metabolites like carotenoids, flavonoids, and phenolics were found to have stimulatory effects on the AhR/XRE signaling pathway. Therefore, the stimulatory effects of the *M. oleifera* glucosinolates on AhR/XRE was also investigated. Furthermore, the genotoxic effects of the hydrolyzed *M. oleifera* glucosinolates were analyzed since examinations of some glucosinolates and their corresponding breakdown products have demonstrated the formation of DNA damage and mutagenic effects (Baasanjav-Gerber et al., 2011a; Glatt et al., 2011; Krehl et al., 2012).

## Materials and methods

### Preparation of *M. oleifera* glucosinolate extracts and standards

Two main glucosinolates of *M. oleifera*, α-4-rhamnopyranosyloxy-benzyl glucosinolate (Rhamno-Benzyl-GS) and acetyl-α-4-rhamnopyranosyloxy-benzyl glucosinolate Isomer III (Ac-Isomer-GS III), and three standards obtained from other plant species, 4-hydroxybenzyl glucosinolate (sinalbin), 4-methylsulfinylbutyl glucosinolate (glucoraphanin), and 3-indolylmethyl glucosinolate (glucobrassicin), were selected for this study. The two *M. oleifera* glucosinolates were fractionated from the methanolic leaf extract from ecotype 4880 (origin in the USA) as described in more detail in Förster et al. (2015b). Briefly, 25 g powdered freeze-dried leaf material was heated up to 80 °C in 300 ml 70% methanol for 10 min. After centrifugation (12,000 *g*, 10 min) the pellet was re-extracted two times with 100 ml 70% methanol. Columns (Telos Filtration Columns, Kinesis, 150 ml) were prepared with DEAE Sephadex solution, imidazole solution and ultra-pure water. The combined supernatants were loaded on the columns. After washing the columns with formic acid:isopropanol:water (ratio 3:2:5) and ultra-pure water, the glucosinolates were precipitated by eluting with 0.5 M potassium sulfate into pure ethanol. The supernatants were dried, re-dissolved in methanol (99.8%), and centrifuged. Ethanol was added to the supernatants and solvents were evaporated to dryness. The resulting crystallized glucosinolate mixture was re-dissolved in ultra-pure water and used for fractionation of single glucosinolates on a Dionex HPLC system equipped with a fraction collector (Dionex FOXY). Glucosinolates were separated on 2.1 x 250 mm AcclaimTM 120 RP18 column (5 µm, Dionex) at 25 °C. Buffered eluents (eluent A: 100% 0.1 M ammonium acetate, B: 40% acetonitrile/0.1 M ammonium acetate) were used for analysis. The eluent program was: 0 - 1 min 0.5% B, 8 - 10 min 20% B, 18 - 19 min 50% B, 28 - 33 min 99% B and 36 - 43 min 0.5% B at a flow rate of 0.4 ml/min and the eluent was monitored at 229 nm. The identity of the *M. oleifera* glucosinolates was proved by mass spectrometry. Rhamno-Benzyl-GS had a purity of > 95%, Ac-Isomer-GS III > 85% (Ac-Isomer-GS I and II ≤ 12%, Rhamno-Benzyl-GS ≤ 2%). A purity of > 85% of Ac-Isomer-GS III had to be accepted because of the instability of this compounds as described in Förster et al. (2015b). The other glucosinolate standards included in the analysis for comparison of bioactivity, sinalbin,

glucoraphanin, and glucobrassicin, were isolated according to the procedure described in Baasanjav-Gerber et al. (2011a).

Different bioassays were performed using the two main leaf glucosinolates of *M. oleifera* in their hydrolyzed form (by adding myrosinase) to analyze (1) potential cytotoxic effects (neutral red uptake (NRU) assay), (2) the protective capacity against inflammatory stimuli and carcinogens (luciferase reporter gene assay, gene expression analysis, enzyme activity analysis), and (3) possible mutagenic effects (Ames test, HPRT assay, SCE assay).

**HepG2 cell culture**

The human hepatocellular carcinoma cell line (HepG2 cells, ATCC HB-8065) was used for the NRU-assay, and reporter gene, gene expression, and the gene activity assays. Cells were grown in RPMI-1640 medium (Gibco, Karlsruhe), supplemented with 2 mM L-alanyl-L-glutamine (Gibco), 10% heat inactivated fetal calf serum (FCS, Biochrom, Berlin), 100 U/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco), pH 7.1, in an incubator (humidified atmosphere) at 37 °C and with 5% $CO_2$. All experiments were repeated three times in triplicate.

**Neutral Red Uptake (NRU) assay**

The cytotoxicity of the *M. oleifera* glucosinolates was analyzed using the NRU assay described in detail in Repetto et al. (2008). Briefly, HepG2 cells were seeded at a density of 4 x $10^4$ cells per well in 96-well plates. After 24 h, the culture medium was removed and cells were treated with a defined concentration of the glucosinolates dissolved in FCS-free culture medium in the presence of myrosinase (3.8 U/ml, Sigma-Aldrich). The final glucosinolate concentrations in wells were 0 (control), 2.5, 5, 10, 20, 30, 40, 50, and 100 µM. After an additional 24 h, the medium was removed and culture medium containing neutral red (40 µg/ml, Sigma-Aldrich) was added for a further 24 h. Then, cells were washed with PBS and neutral red destainer solution (50% (v/v) ethanol, 49% (v/v) sterile water, 1% (v/v) glacial acetic acid). The absorbance was measured photometrically **(**Plate photometer Synergy 2, Biotek Instruments GmbH, Bad Friedrichshall) at a wavelength of 540 nm.

**Luciferase reporter gene assay**

HepG2 cells were seeded in 24-well plates (1.5 x $10^5$cells per well). After 24 h the cells were transfected with pSV-β-galactosidase control vector (0.5 µg/cavity, Promega, Mannheim), TurboFect™ Transfection Reagent (0.65 µl/cavity, Thermo Scientific, St. Leon-Rot), and the relevant reporter plasmid (0.15 µg/cavity, pGL3-basic or pGL3-enhancer, Promega, Mannheim) according to the manufacturer's protocol. Cells were stimulated with 1, 2.5, and 5 µM glucosinolate in the presence of myrosinase (3.8 U/ml; control only myrosinase) for additional 24 h. Then, cells were washed with PBS, scraped off, suspended in 150 µl lysis buffer (5x RLB, Promega, Mannheim), and frozen. Luminescence was determined by analyzing 20 µl of the lysate (Luminoskan Acent, Labsystems, Finland). Luciferase activity was normalized to β-galactosidase activity, determined photometrically at 405 nm (Plate photometer Synergy 2, Biotek Instruments, Bad Friedrichshall) (Haack et al., 2010). Reporter gene assays were performed with specifically modified luciferase reporter plasmids (Table 1). The respective unmodified luciferase plasmids of pGL3-basic and pGL3-enhancer (pGL3 luciferase reporter vector, Promega, Mannheim, Germany) served as controls.

*Plasmid constructions*

Promoter regions of human *NQO1* (NAD(P)H:quinone oxidoreductase 1), *GPx2* (glutathione peroxidase 2), and murine *Srxn1* (sulfiredoxin 1) genes were obtained by PCR from the genomic DNA from HepG2 cells and cloned into the reporter vector pGL3-basic (described in detail for *GPx2* in Banning et al., 2005). To analyze the influence of the ARE and XRE core sequences on the *NQO1* gene promoter activity, the respective promoter sequences of the human *NQO1*-ARE and –XRE (Nioi & Hayes, 2004) were cloned into the reporter vector pGL3-enhancer (pGL3enh-h*NQO1*-ARE, pGL3enh-h*NQO1*-XRE; Table 1). To verify the involvement of ARE and/or XRE, the core sequences of the human *GPx2* promoter were modified by site-directed mutagenesis. The sequences obtained were inserted in the pGL3-basic reporter vector (described in detail in Lippmann, 2013). All reporter plasmids formed the basis for luciferase reporter gene assays.

Tab. 1: Plasmids used in the reporter gene assays in the present study

| Plasmids | Plasmid characteristics | Source |
|---|---|---|
| pSV- β-Gal | Control vector plasmid, β-galactosidase | Promega, Mannheim |
| pGL3-basic | Luciferase reporter gene plasmid without promoter or enhancer | Promega, Mannheim |
| pGL3-enhancer | Luciferase reporter gene plasmid without promoter but with enhancer | Promega, Mannheim |
| pGL3bas-h*NQO1*-1080+20 | 1,099 bp of the human *NQO1* promoter in pGL3-basic | Lippmann, 2013 |
| pGL3bas-h*GPX2*-2111+1 | 2,111 bp of the human *GPx2* promoter in pGL3-basic | Banning et al., 2005 |
| pGL3bas-m*SRXN1*-795+217 | 1,011 bp of the murine *Srxn1* promoter in pGL3-basic | Kim et al., 2010 |
| pGL3enh-h*NQO1*-ARE | pGL3-enhancer with 1 copy of the h*NQO1*-ARE | This study |
| pGL3enh-h*NQO1*-XRE | pGL3-enhancer with 1 copy of the h*NQO1*-XRE | This study |
| pGL3bas-h*GPX2*-mutARE | *GPx2* promoter in pGL3-basic with mutation in the ARE core sequence | Banning et al., 2005 |
| pGL3bas-h*GPX2*-mutXRE | *GPx2* promoter in pGL3-basic with mutations in the XRE core sequence | Haack et al., 2010 |

**Analysis of gene expression**

*RNA-extraction*

HepG2 cells were seeded in 6-well plates (5 x $10^5$ cells per well). The culture medium was removed after 48 h. Starvation medium (1979 µl) and 21 µl of a stock solution containing autoclaved water, the respective glucosinolate (standard 1 mM), and myrosinase (3.8 U/ml) were added. Hence, the cells were stimulated with a glucosinolate concentration of 5 µM. After 8 h the medium was removed and each well was washed with 2 ml PBS twice. Afterwards, the cells were incubated with 0.5 ml trypsin solution per well for 4 min at 37 °C in the incubator. Five ml culture medium was added to each well. Cells were suspended and transferred into a 15 ml centrifugation tube. The tubes

were centrifuged for 5 min at 300 *g* and room temperature. The cell pellet was snap-frozen in liquid nitrogen and stored at -80 °C until RNA extraction. RNA extraction was done according to the protocol in the extraction sets of Qiagen (RNeasy Mini Kit Set, RNAse-free-DNase Set). Twice 20 µl RNAse-free water was added on the filter membrane to elute RNA. The RNA concentration and quality in the samples were determined by using an Agilent RNA 6000 Nano Kit (Agilent Technologies) and Agilent 2100 Bioanalyzer. RNA samples used for analysis had a RNA integrity number (RIN) of 9 - 10.

*Reverse transcription PCR*

Reverse transcription of RNA into cDNA was performed in a thermocycler (Biometra, Göttingen). Three µg RNA (µl calculated by RNA concentration of each sample), 1.5 µl oligo-dT(15) primer (100 µM, Promega Mannheim), and autoclaved water (ad 21 µl) were mixed. Samples were transferred into a thermocycler and the following program was applied: (1) 4 min at 72 °C, (2) 2 h 37 °C, (3) 10 min 94 °C, (4) 10°C. At the beginning of step (2) of the program, 24 µl of the master mix (0.7 nM dNTPs (Bioline, Luckenwalde), 9 µl 5x M-MLV RT buffer (Promega), 0.1 mg/ml BSA (New England Biolabs, Frankfurt a. M.), 0.75 µl rRNAsin® RNase Inhibitor (40 U/µl, Promega), 4 U/µl M-MLV Reverse Transcriptase (Promega), and 9.9 µl autoclaved water) were added to each sample. The cDNA concentration of each sample was adjusted to yield 20 ng/µl.

*Real-time PCR*

To analyze the expression of selected genes 11 µl (12 µl as blank) autoclaved water, 1 µl dissolved cDNA, and 13 µl master mix were added per well in a 96-well plate. The master mix included 2.5 µl TrueStart™ Taq Buffer (10x, Thermo Scientific, St. Leon-Rot), 1.5 µl $MgCl_2$ (25 mM, Thermo Scientific, St. Leon-Rot), 0.5 µl dNTPs (10 mM, Bioline, Luckenwalde), 0.25 µl forward primer (25 mM), 0.25 µl reverse primer (25 mM), 7.625 µl autoclaved water, 0.25 µl SYBR®Green I, and 25 mU/µl TrueStart™ Taq DNA Polymerase (Thermo Scientific) per well. The following real-time PCR program was used: (1) 5 min at 95 °C, (2) 30 s at 95 °C, (3) 30 s at 60 °C, (4) 30 s at 75 °C, (5) 1 min at 95 °C, (6) 30 s for 60 °C (slow temperature increase to 95 °C), (7) 30 s at 95 °C (steps (5) – (7): melting curve). Steps (2) to (4) were repeated 39 times. Measurements were

done with the Mx3005P™ qPCR System (Stratagene, Amsterdam/Netherlands) and the program MXPro qPCR Software 4.10 of Stratagene. The mRNA expression of the genes was analyzed in triplicate and repeated three times. For normalization, two reference genes (*ACTB*, *RPL13A*) were used. The relative quantification was done by a standard curve with purified PCR products ranging from 1 x $10^3$ to 1 x $10^8$ copies (calibration line) of each analyzed gene.

**Enzyme activities**

Next to NQO1, the enzyme activity of TrxR1 was measured. Due to a lack of substrate specificity of GPx2, the analysis gave results for the enzyme activity of all GPx enzyme forms. Therefore, GPx2 was excluded from the enzyme analysis experiment.

Seeded cells ($10^4$ cells per well, 96-well plate) were stimulated with 5 µM sinalbin, glucoraphanin, Rhamno-Benzyl-GS, Ac-Isomer-GSs III and myrosinase (3.8 U/ml) or treated with myrosinase only (control). To measure the influence of selenium on the enzyme activity, sodium selenite (final concentration 50 nM) was added. After three days the cell lysate was prepared as described in detail in Lippmann (2013).

NQO1

NQO1 activity was determined after a protocol of Prochaska & Santamaria (1988). Three µl cell lysate, 50 µl NQO1 inhibition solution (determination of NQO1-independent conversion of 5,5'-dithio-bis (2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid; 0.3 mM dicoumarol in 0.5% DMSO and 0.5 mM potassium phosphate, pH 7.4) or 50 µl water (determination of NQO1-dependent and NQO1-independent conversion), and 197 µl master mix (25 mM Tris/HCl, 655 mg/l BSA, 9.9% Tween 20, 5 µM FAD, 1 mM glucose-6-phosphate, 30 µM NADP, 300 mg/l 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 0.3 mU/µl glucose-6-phosphate dehydrogenase, 50 µM menadione) were mixed. The change in absorption caused by the reduction of MTT by menadione was determined photometrically at 590 nm for a period of 5.5 min (Plate photometer Synergy 2, Biotek Instruments, Bad Friedrichshall). The NQO1-independent conversion (background activity determined in the presence of dicoumarol, an NQO1 inhibitor) was deducted from the total conversion (independent + dependent

conversion determined in the absence of dicoumarol) to determine the NQO1 activity (dependent conversion). Calculation was based on the Lambert-Beer´s law.

TrxR1

The thioredoxin reductases 1 (TrxR1) activity was measured based on the protocols of Amér et al. (1999) and Gromer et al. (2002). Reaction buffer (200 µl; 100 mM potassium phosphate, 2 mM EDTA, pH 7.4) and 15 µl DTNB (50 mM in DMSO) were added to 10 µl cell lysate. The reaction was started by adding 25 µl NADPH (2 mM in reaction buffer). The values of an unspecific conversion of DTNB (reaction without NADPH, independent reaction) were deducted from the determined conversion by adding NADPH (independent + dependent reaction). The increase in absorption was determined photometrically at 412 nm at the first two minutes of the chemical reaction based on the Lambert-Beer´s law. To calculate the enzyme activity [mU/mg protein] the protein content in the cell lysate was determined after a method of Bradford (1987). Ten µl cell lysate and 200 µl staining solution (diluted 1:5, Bio-Rad, München) were incubated for 10 min at room temperature. The extinction was determined photometrically at 630 nm. The protein concentration was quantified by a standard curve of BSA (Protein Assay Standard II, Bio-Rad).

**Genotoxicity assays**

The genotoxicity tests were conducted in the presence of myrosinase, unless specified otherwise. The protocols correspond to those previously used with 1-methoxy-3-indolylmethyl glucosinolate (neoglucobrassicin). This glucosinolate had provided clear positive results in all three test systems (Baasanjav-Gerber et al., 2011b; Glatt et al., 2011).

*Gene mutation assay in Salmonella typhmurium: Ames test*

Mutagenicity was determined using a liquid-preincubation version of the assay described by Maron & Ames (1983) with modifications given in Baasnajav-Gerber et al. (2011a). *Salmonella typhimurium* strains TA100 and TA104, both substitution-mutated strains (TA100: hisG46 mutation; TA104: hisG428 mutation), were used, as they were responsive to the mutagenic action of various glucosinolates (Baasanjav-Gerber et al.,

2011a). In this article the mutagenicity of Rhamno-Benzyl-GS and Ac-Isomer-GS III (final glucosinolate content on plate: 0, 100, 200, 400, 800, 1200, 1800, 2500 nmol) on the two *S. typhimurium* strains was analyzed. Briefly, glass tubes (10 ml) containing 400 µl buffer (150 mM KCl and 10 mM sodium phosphate buffer, pH 6.4), with or without myrosinase (30 mU), and bacterial suspension (100 µl; $5 – 10 \times 10^9$ colony-forming units per ml) were warmed to 37 °C in a water bath for 2 min. Then, the test compound dissolved in 100 µl water was added. After incubation for 60 min at 37 °C, 2 ml warm soft agar (6 mg/ml agar, 6 mg/ml NaCl, 50 µM L-histidine, 50 µM D-biotin, 50 µM L-tryptophan in 25 mM sodium phosphate buffer, pH 7.4) was added. The mixture was poured onto Petri dishes containing 24 ml minimal agar (15 mg/ml agar in Vogel-Bonner E medium with 20 mg/ml glucose). After incubation for 2 – 3 days at 37 °C in the dark, colonies ($his^+$ revertants) were counted. Incubations containing glucosinolates were carried out in triplicate. Higher numbers of replicates were used for the negative controls (9 cultures for TA100 and 6 cultures for TA104). The result was classified positive, if the number of the revertants (mean value at any dose level) was increased at least 2-fold in strain TA100 or 1.7-fold in strain TA104 (which has a higher spontaneous mutation rate) with a plausible dose–response relationship. It was also classified positive if the increase was at least 1.5-fold (TA100) or 1.3-fold (TA104) and confirmed in a repeat experiment.

*HPRT gene mutation assay in V79 cells: HPRT assay*

Chinese hamster V79 cells were grown in Dulbecco´s modified Eagle medium (DMEM, Gibco), supplemented with fetal bovine serum (5%, Sigma-Aldrich), penicillin (100 units/ml, Gibco), and streptomycin (100 µg/ml, Gibco). The cultures were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$. Acquisition of resistance towards 6-thioguanine (involving inactivation of the X-chromosomal *HPRT* gene) was used to study the induction of gene mutations in V79-derived cell lines. A detailed protocol for the mutagenicity assay has been described elsewhere (Glatt et al., 1991). The protocol was slightly modified to the needs of glucosinolates. Briefly, $1.5 \times 10^6$ V79 cells were added to 30 ml medium (DMEM) in a 15 cm Petri dish. After 18 h, myrosinase (10 mU/ml, final concentration) and the test compound (dissolved in 100 µl water, or water only) was added. Eight negative control cultures and four cultures

for each glucosinolate concentration were used: Rhamno-Benzyl-GS (0, 25, 50, 75, 100 µM). The exposure was terminated after 72 h, and the cells were detached by treatment with trypsin. Their number, expressed as a percent of the corresponding value of the solvent control cultures, was used as a measure of the cytotoxicity of the treatment. The cells were then subcultured (3 x $10^6$ cells per plate) in normal medium for 3 days and then subcultured again using 6-thioguanine-supplemented (7 µg/ml) medium for the selection of mutant ($10^6$cells per15 cm Petri dish, six dishes) and normal medium for determining the total number of colony-forming cells (100 cells per 6 cm Petri dish, three dishes.). After 12 days, the cultures were fixed in ethanol and stained with Giemsa. The colonies were counted and mutant frequencies were calculated for each original culture. The results are evaluated following standard criteria of the laboratory. A test is considered negative if the mean mutant frequency of no group treated with any concentration of the test compound was increased by ≤ 10 mutants per $10^6$ cells. It is considered positive if the treatment with at least one concentration of the test compound led to an increase in mutant frequency by ≥ 40 x $10^{-6}$ with a plausible concentration–response curve. In all other cases, the evaluation is based on statistical analysis ($p < 0.05$, Students t-test) if the number of cultures per group is ≥ 4 (which was the case in the present study) or repeat experiments are conducted (if the number of cultures per treatment group is low, 2 - 3).

*Induction of sister-chromatid exchange in V79 cells: SCE-assay*

The assay was performed as described previously (Glatt et al., 2005) with minor modification. A total of $1.67 \times 10^5$ V79 cells and 10 ml medium were added to each 25 $cm^2$ Petri dish. After 19 h, myrosinase (10 mU/ml, final concentration), the test compound (dissolved in 100 µl of 100 mM ammonium acetate in water, or the solvent only) and 5-bromo-2-deoxyuridine (5 µg/ml) were added. The final concentrations of the glucosinolates were 0, 10, 30, 100 and 300 µM. Two separate cultures were used for each treatment. After a further 32-h period, colcemid was added in a final concentration of 0.25 µg/ml. Four hours later, the cells were harvested, treated with hypotonic KCl solution (40 mM) for 20 min at room temperature, fixed in methanol:acetic acid (3:1, v/v), stored at 4 °C overnight, and dropped on ice-cold glass slides. The slides were air-dried and stained according to the fluorescence plus Giemsa technique. A total of 25 metaphases containing 20 to 23 chromosomes were scored for SCE per culture. The

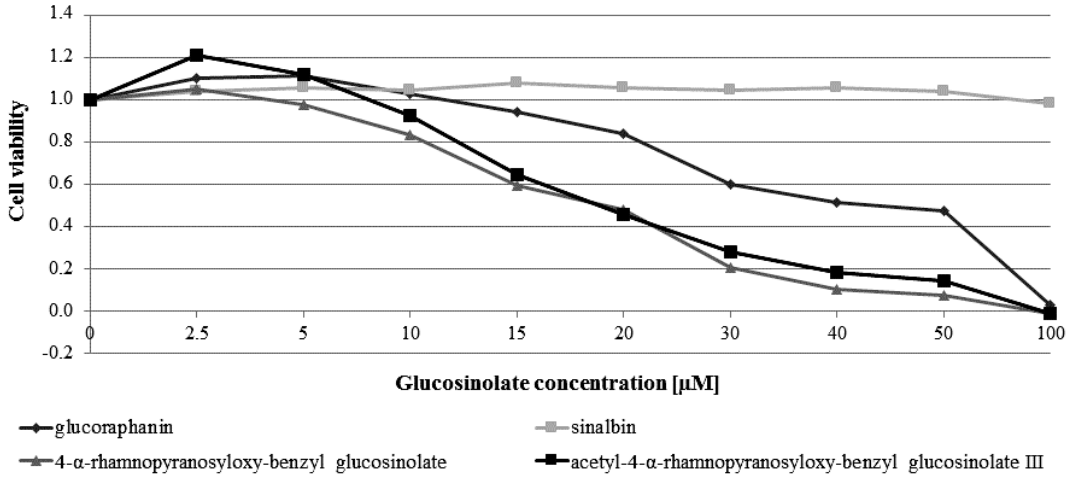
number of SCE per metaphase was standardized to 22 chromosomes. The results for both parallel plates were pooled. The frequency of SCE in treatment groups was compared with that observed in the solvent controls using the Mann-Whitney U test. Concurrently, the proliferation index (proportion of cells in first division + 2 × proportion of cells in second division + 3 × proportion of cells in higher than second division) was determined and used as a measure for cell cycle delay.

## Results

The different assays were conducted in the presence of myrosinase, unless specified otherwise. Thus, the glucosinolates were hydrolyzed enzymatically *in situ*.

### Cell viability measured by NRU assay

HepG2 cells were stimulated with hydrolyzed sinalbin, glucoraphanin, Rhamno-Benzyl-GS, or Ac-Isomer-GS III in the presence of myrosinase. The NRU assay revealed a strong influence of aromatic *M. oleifera* glucosinolates as well as the aliphatic 4-methylsulfinylbutyl glucosinolate on cell viability (Figure 1). The cell viability was significantly decreased at concentrations higher than 15 µM hydrolyzed *M. oleifera* glucosinolates. For glucoraphanin, concentrations from 20 µM significantly decreased cell viability, whereas hydrolyzed sinalbin had no influence on the cell viability of HepG2 cells at concentrations up to 100 µM (Figure 1). Overall *M. oleifera* glucosinolates exhibit higher toxic effects compared to glucoraphanin. The $EC_{50}$ values of Rhamno-Benzyl-GS and Ac-Isomer-GS III were 18.4 µM and 17.4 µM, respectively, whereas the value for glucoraphanin was 49.3 µM.




| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| ◆ | ab | a | a | ab | bc | c | d | d | d | e |
| ■ | a | a | a | a | a | a | a | a | a | a |
| ▲ | a | a | a | ab | bc | cd | de | e | e | e |
| ■ | b | a | a | b | c | d | e | ef | f | g |

Different letters indicate significant differences in cell vitality between different concentrations of each glucosinolate, ANOVA, Tukey's HSD test, $p \leq 0.05$

Fig. 1: Viability of HepG2 cells treated with different concentrations of four hydrolyzed glucosinolates

**Luciferase reporter gene assays**

According to the NRU-assay, the concentration of 5 µM was identified as the highest possible concentration for usage with no influence of the four tested glucosinolates on the viability of HepG2 cells. Therefore, a concentration of 5 µM was chosen for the reporter gene assays performed. The stimulation of the promoter regions of the genes encoding for *NQO1*, *GPx2*, and *Srxn1* by hydrolyzed glucosinolates was analyzed. All three gene promoter activities were significantly increased by adding glucoraphanin, Rhamno-Benzyl-GS, or Ac-Isomer-GS III (Figure 2). Here, the activities of both, *NQO1* and *Srxn1*, promoters increased with increasing concentrations of glucoraphanin, Rhamno-Benzyl-GS, and Ac-Isomer-GS III up to 5 µM (significant differences shown in Figure 2, lower case letters). In the case of *GPx2*, the maximum promoter activity was already reached at 2.5 µM. In contrast, sinalbin did not show any influence on the activities of the different gene promoters. For each concentration level, the *Moringa* glucosinolates led to

a higher increase in promoter activity, which in most cases was significant, followed by glucoraphanin and sinalbin (significant differences shown in Figure 2, capital letters).

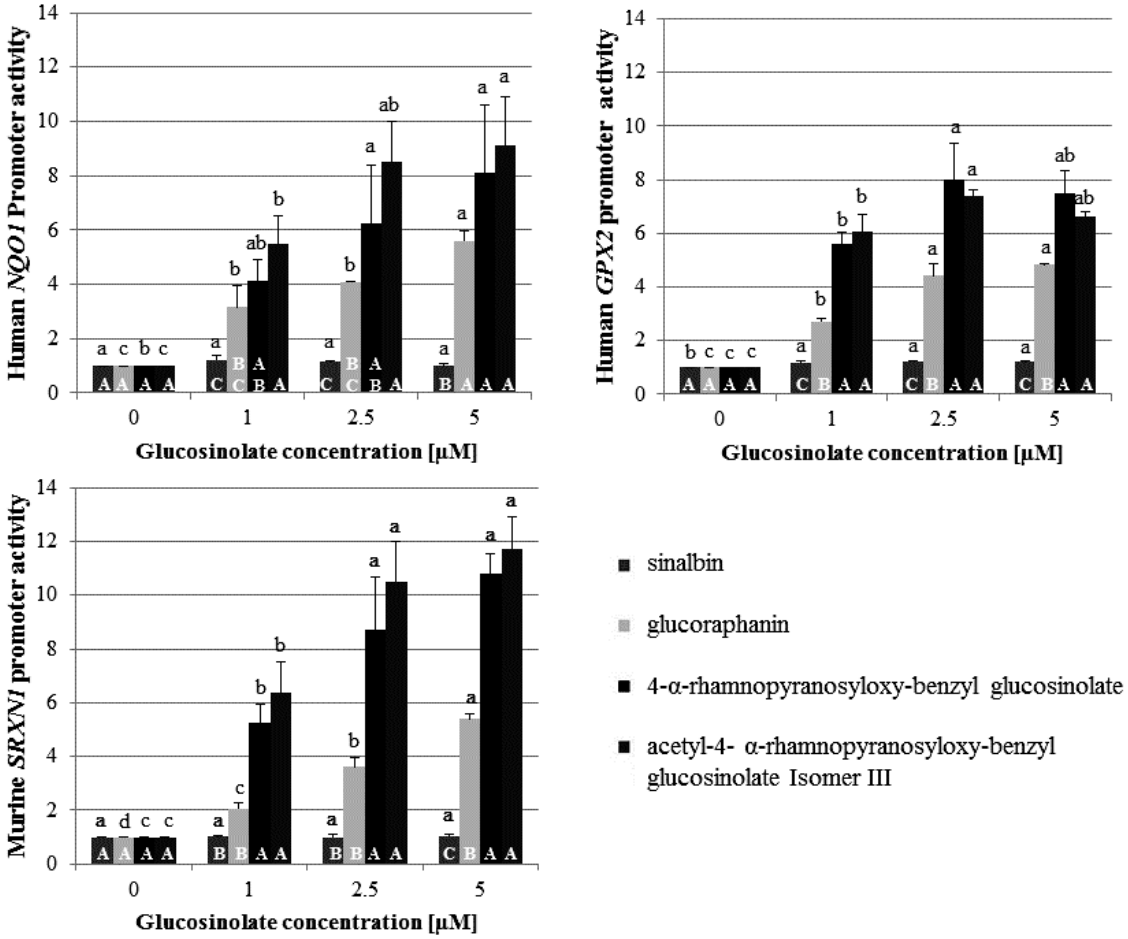



Fig. 2: Stimulating effect of four hydrolyzed glucosinolates on the entire promoters of NAD(P)H:quinone oxidoreductase 1 (*NQO1;* pGL3bas-h*NQO1*-1080+20), glutathione peroxidase 2 (*GPx2;* pGL3bas-h*GPX2*-2111+1), and sulfiredoxin 1 (*Srxn1;* pGL3bas-m*SRXN1*-795+217) (Tukey's HSD test, $p \leq 0.05$, lower case letters: significant differences between concentrations within one glucosinolate, capital letters: significant differences between glucosinolates within one concentration)

Since the analyzed genes are Nrf2 target genes, the extent to which the selected ARE motif of the *NQO1* promoter had an influence on the promoter activity was examined. The XRE motif was also included in the analysis to clarify whether the effects of *Moringa* glucosinolates on promoter activities were mediated through the XRE motif as an

indicator for a stimulation of the aryl hydrocarbon receptor (AhR) signaling pathway. A stimulatory effect of hydrolyzed glucoraphanin and the *M. oleifera* glucosinolates via the *NQO1*-ARE element was observed; however, in the case of Ac-Isomer-GS III the increase was not significant (Figure 3). Glucobrassicin had no influence on the promoter activity mediated by the *NQO1*-ARE element when compared to the control. None of the tested glucosinolates stimulated the *NQO1*-XRE element. Thus, the *M. oleifera* glucosinolates activated the *NQO1* promoter primarily via the ARE motif.

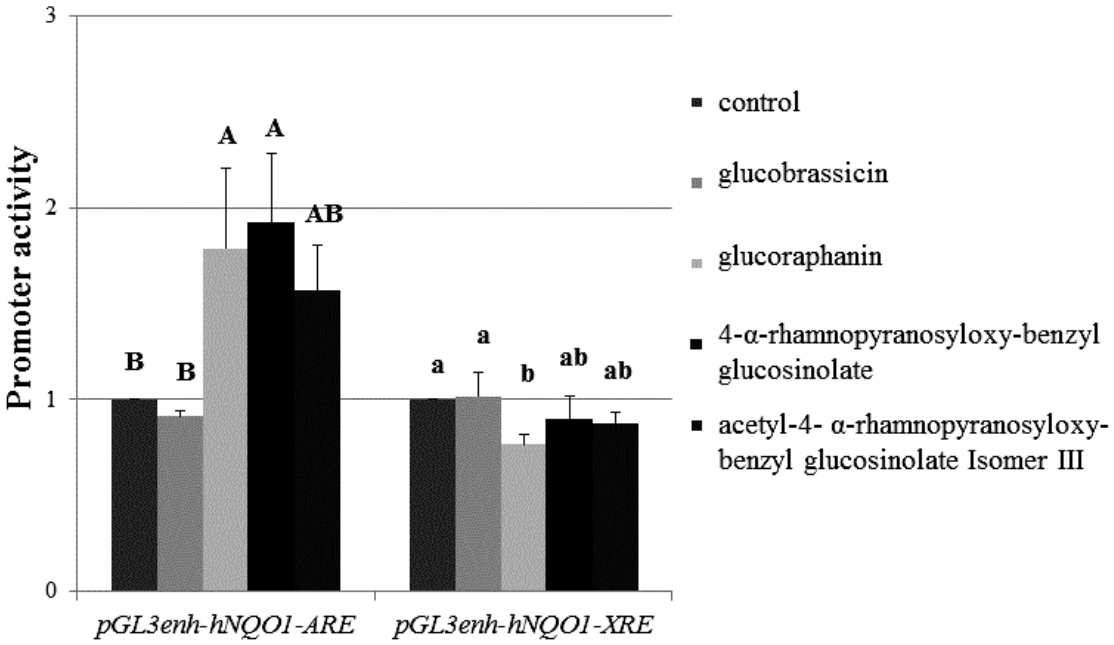



Fig. 3: Stimulating effect of 5 µM of four hydrolyzed glucosinolates on the luciferase promoter activity via the ARE or XRE motif of the NAD(P)H:quinone oxidoreductase 1 (*NQO1*) gene promoter alone (*pGL3enh-hNQO1-ARE*, *pGL3enh-hNQO1-XRE*); Tukey´s HSD test, $p \leq 0.05$, capital letters: significant differences between different glucosinolates within pGL3enh-hNQO1-ARE, lower case letters: significant differences between different glucosinolates within pGL3enh-hNQO1-XRE

To investigate whether docking of the response factor on the response element can be prevented, the pGL3-basic vectors with a mutation in the ARE or XRE motif (Table 1) were used for further studies. In comparison with the stimulating effects of the glucosinolates on the wild type *GPx2* promoter, the mutated ARE significantly prevented

stimulation of the *GPx2* promoter by all glucosinolates tested (Figure 4, lower case letters, Tukey´s HSD test, $p \leq 0.05$). The reduction of promoter activity was significantly higher in ARE mutated promoters in comparison to XRE mutated promoters for all glucosinolates tested. All glucosinolates enhanced the promoter activity in the reporter genes including the whole *GPx2* promoter and including the *GPx2* promoter with the mutated XRE motif (Figure 4, capital letters, Tukey´s HSD test, $p \leq 0.05$). Thus, the ARE motif had a much stronger influence on *GPx2* promoter activity than the XRE motif.

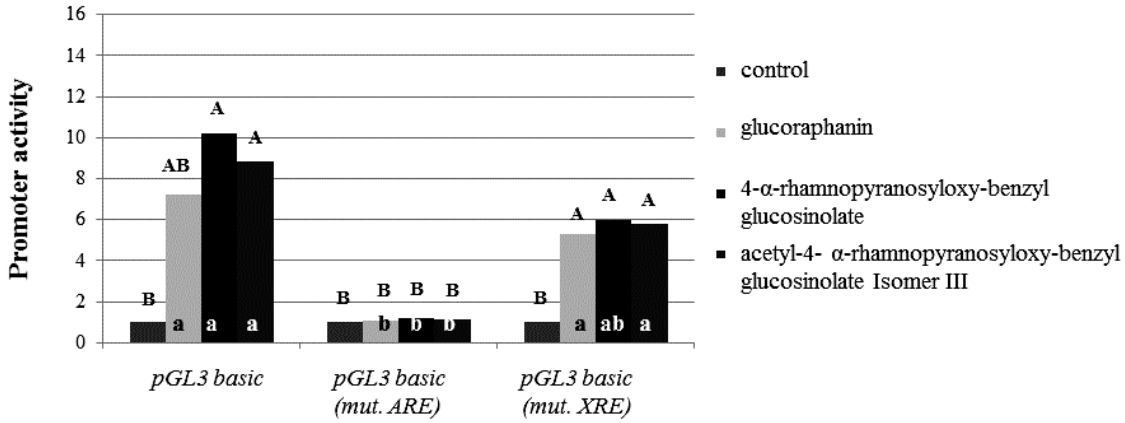



Fig. 4: Stimulating effect of four hydrolyzed glucosinolates on the promoter activity of glutathione peroxidase 2 (*GPx2*; pGL3bas-hGPX2-2111+1), of a *GPx2* promoter with a mutated ARE motif (pGL3bas-hGPX2-mutARE), and a *GPx2* promoter with a mutated XRE motif (pGL3bas-hGPX2-mutXRE). (one-way ANOVA followed by Tukey´s HSD test, capital letters: significant differences between different glucosinolates within on vector, lower case letters: significant differences between different vectors within one glucosinolate, $p \leq 0.05$)

**Analysis of RNA expression by real-time PCR**

The influence of *M. oleifera* glucosinolates on the expression of different selected genes was also investigated (Table 2). Here, in addition to *NQO1*, *GPx2*, and *TrxR1* as Nrf2 target genes, the expression of three different genes from the CYP gene family as well as genes for transcriptions factors or cancer influencing genes were analyzed. Glucoraphanin, Rhamno-Benzyl-GS, and Ac-Isomer-GS III significantly increased the expression of *NQO1*, *GPx2*, *TrxR1*, and *Keap1* in HepG2 cells (Table 2). No significant

effect was detected for *Nrf2*, *AhR*, and *CYP1A1*. The expression of *CYP3A4* and *CYP2E1* was significantly decreased by Rhamno-Benzyl-GS and Ac-Isomer-GS III.

Tab. 2: Expression (on RNA level) of different genes in HepG2 cells after incubation with 5 µM of four hydrolyzed glucosinolates

| | **Control** | **Sinalbin** | **Glucoraphanin** | **Rhamno-Benzyl-GS** | **Ac-Isomer-GS III** |
|---|---|---|---|---|---|
| **Nrf2 target genes** | | | | | |
| ***NQO1*** | 1(a) | 1.10(a) | 2.33(b) | 2.48(b) | 2.50(b) |
| ***GPx2*** | 1(a) | 1.10(a) | 1.85(b) | 1.98(b) | 1.82(b) |
| ***TrxR1*** | 1(a) | 1.16(a) | 6.99(b) | 9.36(b) | 8.13(b) |
| | | | | | |
| **CYP gene family** | | | | | |
| ***CYP3A4*** | 1(ab) | 1.15(a) | 0.69(bc) | 0.62(c) | 0.64(c) |
| ***CYP1A1**** | 1(a) | 1.21(a) | 1.03(a) | 1.00(a) | 0.85(a) |
| ***CYP2E1**** | 1(a) | 0.81(ab) | 0.75(ab) | 0.64(b) | 0.79(b) |
| | | | | | |
| ***Keap1*** | 1(b) | 0.98(b) | 1.58(a) | 1.76(a) | 1.44(a) |
| ***Nrf2*** | 1(a) | 0.98(a) | 1.09(a) | 1.06(a) | 1.00(a) |
| ***AhR*** | 1(a) | 0.97(a) | 1.09(a) | 1.46(a) | 1.40(a) |

* low averaged number of copies
significant differences within each gene, Tukey´s HSD test, $p \leq 0.05$
GS = glucosinolate, Rhamno-Benzyl-GS = α-4-rhamnopyranosyloxy-benzyl glucosinolate, Ac-Isomer-GS III = acetyl-α-4-rhamnopyranosyloxy-benzyl glucosinolate Isomer III

**Enzyme activity**

Glucoraphanin, Rhamno-Benzyl-GS, and Ac-Isomer-GS III increased the activity of NQO1 and TrxR1 in HepG2 cells (Figure 5). The activity of both enzymes was significantly higher than in the control and in cells incubated with sinalbin. Being a selenoprotein, the activity of TrxR1 was dependent on the availability of selenium, whereas the addition of sodium selenite did not show any effect on the activity of NQO1.

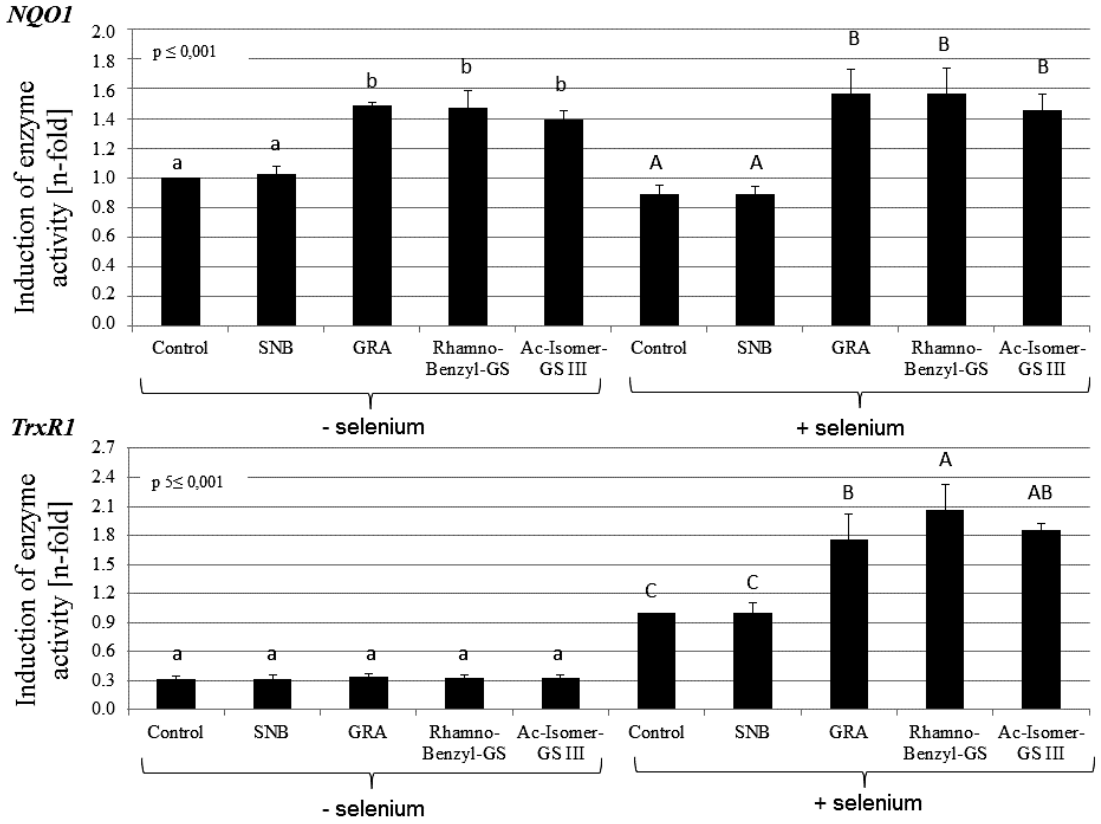



Fig. 5: Stimulating effect of hydrolyzed sinalbin (SNB), glucoraphanin (GRA), α-4-rhamnopyranosyloxy-benzyl glucosinolate (Rhamno-Benzyl-GS), and acetyl-α-4-rhamnopyranosyloxy-benzyl glucosinolate Isomer III (Ac-Isomer-GS III) on the activity of NAD(P)H:quinone oxidoreductase 1 (NQO1) and thioredoxin reductases (TrxR1) in HepG2 cells +/- selenium (sodium selenite; 50 nM). (Tukey's HSD test, $p \leq 0.001$, significant differences within enzyme and selenium treatment and between glucosinolates)

**Genotoxicity investigations**

In general, relatively high quantities of test compounds are required in genotoxicity tests if maximally tolerated concentrations are used. Since the amounts of purified Rhamno-Benzyl-GS and Ac-Isomer-GS III were limited, some compromises had to be made. For this reason, in the first experiment (Suppl. Figure 1) total *M. oleifera* glucosinolate extract was used, while single glucosinolates were used for in-depth analysis (Figure 6, Tables 2 and 3).

Thus, the initial bacterial mutagenicity test, conducted in *S. typhimurium* TA104, was carried out with a mixture of Rhamno-Benzyl-GS and Ac-Isomer-GS III (molar ratio 4:1) (Suppl. Figure 1). A total of 15 different dose levels, ranging from 30 - 3000 nmol/plate, were used. The *M. oleifera* glucosinolates did not show any indication of any mutagenic activity in strain TA104. However, they demonstrated clear bacteriotoxicity at doses ≥ 1000 nmol per plate in the presence of myrosinase, as indicated by a decrease in the number of revertant colonies ($his^+$ colonies) and microcolonies in the background lawn ($his^-$ surviving cells) compared to the negative control. The subsequent experiment in strain TA100, found to be more responsive for many glucosinolates (Baasanjav-Gerber et al., 2011a), was conducted with the separate *M. oleifera* glucosinolates, Rhamno-Benzyl-GS and Ac-Isomer-GS III. The selected dose was based on the results in strain TA104, in particular the toxicity findings. Therefore, the dose range was narrowed to 100 – 2500 nmol per plate. There was no indication of mutagenicity of Rhamno-Benzyl-GS and Ac-Isomer-GS III in strain TA100 (Figure 6). Again bacteriotoxicity was noticed at high doses of each compound (≥ 1800 nmol/plate) in the presence of myrosinase. Sinalbin was concurrently used as a positive control compound (Figure 6, right panel). As in our previous study (Baasanjav-Gerber et al., 2011b), it was mutagenic in the presence, but not in the absence of myrosinase.

Then, genotoxicity tests were conducted in mammalian target cells. The SCE test has the advantage that only modest numbers of cells and incubations volumes (and therefore quantities of test compound) are required. Moreover, many genotoxicants provide a positive result for this endpoint even at very low concentrations (Glatt et al., 2005). 1-Methoxy-3-indolylmethyl glucosinolate (Glatt et al., 2011) and various other glucosinolates, including glucotropaeolin and glucoraphanin (H. R. Glatt, unpublished result) had demonstrated positive results in the SCE test in V79 cells. However, Rhamno-Benzyl-GS and Ac-Isomer-GS III gave negative results, when tested up to the limits given by toxicity (Table 3).

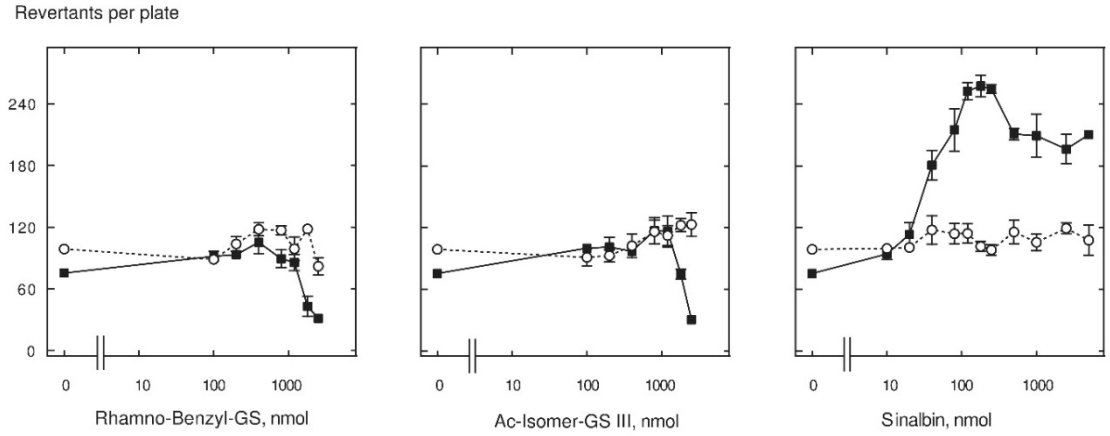



Fig. 6: Mutagenicity test with *Moringa oleifera* glucosinolates and sinalbin in *Salmonella typhimurium* TA100 (presence (■) and absence (O) of myrosinase, 10 mU; α-4-rhamnopyranosyloxy-benzyl glucosinolate (Rhamno-Benzyl-GS); acetyl-α-4-rhamnopyranosyloxy-benzyl glucosinolate Isomer III) (Ac-Isomer-GS III)

Tab. 3: SCE test with *Moringa oleifera* glucosinolates in Chinese hamster V79 cells

| Treatment [a] | Proliferation index [b] | SCE per metaphase [c] |
|---|---|---|
| Negative control | 2.04 ± 0.01 | 6.3 ± 0.4 (50) |
| Rhamno-Benzyl-GS | | |
| 10 µM | 2.16 ± 0.05 | 8.1 ± 0.5 (50) [ns] |
| 30 µM | 1.98 [d] | 9.3 ± 1.0 (25 [d]) [ns] |
| 100 µM | – [e] | – [e] |
| 300 µM | – [e] | – [e] |
| Ac-Isomer-GS III | | |
| 10 µM | 2.12 ± 0.03 | 7.0 ± 0.4 (50) [ns] |
| 30 µM | 2.05 ± 0.01 | 6.4 ± 0.4 (50) [ns] |
| 100 µM | – [e] | – [e] |
| 300 µM | – [e] | – [e] |

[a] Myrosinase (10 mU/ml) was present in all treatment in groups.
[b] Proliferation index (determined from 100 metaphases per culture), mean ± half range of two cultures.
[c] Number of SCE per metaphase (normalised to 22 chromosomes) ± SE of indicated number of metaphases. ns, not statistically different from negative control (Wilcoxon's rank test, $p > 0.05$).
[d] Due to a technical error only one culture could be evaluated.
[e] Treatment with 100 µM or 300 µM Rhamno-Benzyl-GS and Ac-Isomer-GS III was very toxic. Insufficient cells were available for determining proliferation index or SCE frequencies.
α-4-Rhamnopyranosyloxy-benzyl glucosinolate (Rhamno-Benzyl-GS)
Acetyl-α-4-rhamnopyranosyloxy-benzyl glucosinolate Isomer III (Ac-Isomer-GS III)

The HPRT gene mutation test in V79 cells requires much higher quantities of test compound than the SCE test. Therefore, it could only be performed with Rhamno-Benzyl-GS. The result was clearly negative (Table 4).

Tab. 4: HPRT gene mutation test with 4-α-rhamnopyranosyloxy-benzyl glucosinolate (Rhamno-Benzyl-GS) in Chinese hamster V79 cells

| Rhamno-Benzyl-GS, µM [a] | Relative cell number at subculture after treatment, % [b] | Mutants per $10^6$ cells [c] |
|---|---|---|
| | *Experiment 1* | |
| 0 | 100 ± 10 (8) | 1.4 ± 0.5 (8) |
| 6.25 | 100 ± 3 (4) | 2.4 ± 0.7 (4) [ns] |
| 12.5 | 114 ± 2 (4) | 3.5 ± 0.7 (4) [*] |
| 25 | 111 ± 11 (4) | 3.3 ± 2.2 (4) [ns] |
| 50 | 79 ± 5 (4) | 2.9 ± 2.6 (3 [d]) [ns] |
| | *Experiment 2* | |
| 0 | 100 ± 3 (8) | 5.1 ± 0.8 (8) |
| 25 | 93 ± 7 (4) | 31 ± 0.7 (4) [ns] |
| 50 | 88 ± 1 (4) | 3.3 ± 0.6 (4) [ns] |
| 75 | 64 ± 3 (4) | 6.2 ± 2.5 (4) [ns] |
| 100 | 41 ± 11 (4) | 7.4 ± 1.4 (4) [ns] |

[a] Myrosinase (10 mU/ml) was present in all groups.
[b] The mean number of cells in the control culture was set to 100%. Values are mean ± SE of the number of cultures indicated in parentheses.
[c] Values are mean and SE of the number of cultures indicated in parentheses. Results of the treatment groups were statistically compared with the negative control group using Student's t-test: * $0.01 > p > 0.05$; ns, not statistically different from negative control ($p > 0.05$).
[d] Due to a technical error only three cultures could be evaluated.

## Discussion

The NRU, reporter gene, gene expression, and enzyme activity assays provided evidence that hydrolyzed Rhamno-Benzyl-GS and Ac-Isomer-GS III from *M. oleifera* exert cytotoxic effects on HepG2 cells and may have a potential to protect against inflammatory stimuli and carcinogens. While Nrf2-target genes (*NQO1*, *GPx2*, *TrxR1*) were up-regulated and genes of the CYP gene family were not influenced or down-regulated, other genes, related to play a role in carcinogenesis, were not significantly affected (*Nrf2*, *AhR*) or up-regulated (*Keap1*). The Ames test did not indicate any mutagenicity of Rhamno-Benzyl-GS and Ac-Isomer-GS III but demonstrated bacteriotoxicity at high doses in the

presence of myrosinase. SCE and HPRT assays provided no indication for genotoxicity of the hydrolyzed *M. oleifera* glucosinolates in mammalian target cells.

Whereas some glucosinolates show adverse effects, the glucosinolates of *M. oleifera* did not. Baasanjav-Gerber et al. (2011a) described mutagenic and bacteriotoxic effects of various glucosinolates (including neoglucobrassicin and sinalbin) in *S. typhimurium* strains in the presence of myrosinase. All analyzed glucosinolates (including glucoraphanin) tested by Baasanjav-Gerber et al. (2011a) formed DNA adducts; however single, weak adduct spots were detected with aliphatic as well as aromatic glucosinolates and highest adduct levels were found for indole glucosinolates. Detected DNA adducts are formed by complexing of reactive metabolites of genotoxic substances with DNA resulting in lesions which could lead to DNA mutations. Furthermore, other factors like the stage of cancer or selenium availability were able to influence the effects of glucosinolates. Krehl et al. (2012) observed a toxic potential of sulforaphane when selenium was restricted, attributing to a missing antagonism by selenoproteins like GPx2. Higher number of tumors, increased apoptosis in the colonic crypt, as well as higher inflammations scores were found in azoxymethane/dextran sodium sulfate-treated mice if sulforaphane was applied under limited selenium supply compared to selenium adequacy (Krehl et al., 2012).

The NRU assay revealed that the two *M. oleifera* glucosinolates significantly reduced the cell viability of HepG2 cells when their concentrations exceeded 15 µM (Figure 1). Therefore, a maximum concentration of 5 µM was used for analysis of gene expression and enzyme activity to avoid cell damaging effects in the bioassays.

### *M. oleifera* glucosinolates stimulate the Nrf2 pathway

Based on previous findings that hydrolyzed glucoraphanin-derived sulforaphane is a potent inducer of *NQO1* and *GPx2*, it was proposed that Rhamno-Benzyl-GS and Ac-Isomer-GS III have similar effects. Indeed, hydrolyzed Rhamno-Benzyl-GS and Ac-Isomer-GS III stimulated the promoters of *NQO1*, *GPx2*, and *Srxn1* with an efficacy even higher than that of the positive control glucoraphanin (Figure 2), indicating a putative capacity of hydrolyzed *M. oleifera* glucosinolates to stimulate the Nrf2 pathway. In contrast, the promoters of the mentioned genes were not activated by hydrolyzed sinalbin.

Furthermore, no stimulating effects were identified for the intact glucosinolates of *M. oleifera* (without adding myrosinase), as also found for glucoraphanin (Haack et al., 2010), since only hydrolyzed (activated by myrosinase) glucosinolates become bioactive. Whereas our examinations reveal an increase in RNA levels of the Nrf2 target genes *NQO1, GPx2, and TrxR1*, the RNA of Nrf2 itself was not influenced by *M. oleifera* glucosinolates (Table 2). One explanation could be a higher export rate of Nrf2 negative regulators out of the nucleus (Niture et al., 2014) or a modification of reactive cysteines in Keap1 which prevents degradation of Nrf2 and in consequence stabilizes newly synthesized Nrf2 (Kasper et al., 2009). In detail, a modification of cysteine thiol groups could change the confirmation of Keap1 leading to a sole binding of Nrf2 on the ETGE motif (normally two-sites binding of Nrf2 via DLG and ETGE motif). This leads to a prevention of Nrf2 for ubiquitination and therefore stabilization (Brigelius-Flohé et al., 2011). Normally, inducers, like sulforaphane, could react with cysteines and cause dissociation of the Nrf2/Keap1 complex thereby ensuring the translocation of Nrf2 into the nucleus (Dinkova-Kostova et al., 2002). Furthermore, the gene expression of *Keap1* was increased significantly by the *M. oleifera* glucosinolates (Table 2), possibly resulting in a preservation of Nrf2 via the Nrf2/Keap1 complex at an increased cytosolic level, in our opinion. Interestingly, *Keap1* has an ARE in its promoter, known as an autoregulatory loop (Lee et al., 2007). Therefore, it is conceivable that dissociated Nrf2 binds not only on the promoter of *NQO1* and other target genes, but also induces transcription of *Keap1*.

### *M. oleifera* glucosinolates activate the expression of Nrf2 targets via the ARE motif, but not XRE

Both *M. oleifera* glucosinolates stimulate the reporter promoter driven by the ARE motif of *NQO1* (pGL3enh-h*NQO1*-ARE), but not via the isolated *NQO1* XRE motif (pGL3enh-h*NQO1*-XRE; Figure 3). The XRE motif seems not to be necessary for activation of the expression of Nrf2 target genes. Furthermore, if ARE was mutated in the *GPx2* promoter (pGL3bas-h*GPX2*-mutARE), none of the tested glucosinolates was able to stimulate the expression of the reporter gene (Figure 4). This shows that no other motif can compensate for the loss of ARE, indicating again that XRE may play no or a less important role. In addition, mutation of the XRE element (pGL3bas-h*GPX2*-mutXRE) leads to a slightly but not significantly lower stimulation of the *GPx2* promoter (Figure 4). Thus again, the

XRE present in the *GPx2* promoter might not be involved in its stimulation by the *M. oleifera* glucosinolates.

Stimulation of the respective promoter by the ARE motif alone was significant (Figure 3), but not as high as with the entire *NQO1* promoter (Figure 2). These results suggest the involvement of probably multiple ARE motifs as also postulated by Nioi & Hayes (2004) for the promoter of *NQO1*, and by Banning et al. (2005) for that of *GPx2*. However, just one motif shows the core sequence and was therefore used in our analysis. Nonetheless, it is conceivable that similar ARE motifs interact with the Nrf2/Maf complex to mediate an enhanced promoter activity. This has to be further investigated.

Based on the described results, an interaction of Nrf2 and AhR is not involved in the *M. oleifera* glucosinolates-mediated activation of the measured targets. In contrast, Haack et al. (2010) observed a negative crosstalk, as far as the glucoraphanin mediated stimulation of the promotors of *NQO1* and *GPx2* (ARE/Nrf2) was inhibited by hydrolysis products of neoglucobrassicin (XRE/AhR). Kalthoff et al. (2010) showed an induction of UDP-glucoronosyltransferases (UGTs) via AhR (by xenobiotics) and via Nrf2 (by oxidative metabolites). The authors demonstrated a functional interaction of Nrf2 and AhR, since gene induction of *UGT1A8* and *UGT1A10* caused by *ter*t-butylhydroquinone (Nrf2) and TCDD (2,3,7,8-tetrachlordibenzo-*p*-dioxin; AhR) was completely lost by a knockdown of Nrf2 or AhR. Also Bonnesen et al. (2001) reported an interaction between AhR and Nrf2. To obtain a TCDD mediated induction of *NQO1* by TCDD, AhR has to be present. They conclude a formation of a protein complex of TCDD with AhR and Nrf2.

In contrast to our findings with the glucosinolates of *M. oleifera*, other glucosinolates have stimulatory effects on the XRE/AhR signaling pathway. Metabolites of neoglucobrassicin, e. g. *N*-methoxyindolyl-3-carbinol, inhibited hydrolyzed glucoraphanin-induced activation of the *NQO1* or *GPx2* promoter mediated by the activation of an XRE binding site in the promoters (Haack et al., 2010). Furthermore, in contrast to aromatic glucosinolates like the *M. oleifera* glucosinolates and aliphatics like glucoraphanin, indole glucosinolates were found to induce *CYP1A* enzymes, members of the CYP superfamily (Bonnesen et al., 1999; Wang et al., 2013). These phase I (and AhR induced) enzymes metabolize some procarcinogens into ultimate carcinogens and, thus,

are associated with carcinogenesis. Such a function can be ruled out for the *M. oleifera* glucosinolates investigated here, at least for the XREs present in *NQO1* and *GPx2*. This conclusion is reinforced by the results of the gene expression analysis of the *M. oleifera* glucosinolates (Table 2). The RNA expression of *CYP3A4* and *CYP2E1* significantly decreased when cells where stimulated with hydrolyzed glucoraphanin, Rhamno-Benzyl-GS, as well as Ac-Isomer-GS III. The expression of *CYP1A1* was not significantly influenced. These results seem to be comprehensible as far as various studies found that glucoraphanin-derived sulforaphane reduces CYP activity on RNA or enzymatic level or decreases former induced CYP activity (reviewed by Elbarbry & Elrody, 2011). Nakajima et al. (2001) showed an inhibiting effect on diverse members of the CYP gene family by gluconasturtiin derived phenylethyl isothiocyanate. Skupinska et al. (2009) further conclude that the translocation of AhR into the nucleus (and therefore the expression of AhR target genes like the CYPs) can be blocked by isothiocyanates. Based on the finding that the mRNA level of CYPs did not change but protein level did, an alternative assumption is that other intracellular molecules, for example estrogen receptors, can regulate CYP activity (Han et al., 2005; Skupinska et al., 2009). Hence, it is not surprising that the transcription of AhR was not influenced significantly by glucoraphanin and the *M. oleifera* glucosinolates (Table 2).

Second to the fact that the influence of glucosinolate hydrolysis products on enzyme activity depends on the type of cell and enzyme, the isothiocyanate structure seems to be crucial for their stimulating effects (Conaway et al., 1996; Skupinska et al., 2009). Experiments performed by Skupinska et al. (2009) demonstrated that a structural modification of sulforaphane can influence the inhibitory potential on CYP enzyme activity. 2-Oxohexyl isothiocyanate and 5-methylsylfinyl-*n*-amyl isothiocyanate, slightly chemically modified molecules in comparison to sulforaphane, decreased the CYP1A1 activity much stronger than sulforaphane. For 2-oxohexyl isothiocyanate, this was attributed to a change in the electrostatic properties (Skupinska et al., 2009). Additionally, an elongation of the $CH_2$ chain in the isothiocyanate molecule – as demonstrated by 5-methylsylfinyl-*n*-amyl isothiocyanate - leads to an increased inhibitory potency (Conaway et al., 1996; Skupinska et al., 2009). Based on findings from literature, sinalbin, glucoraphanin, and the *M. oleifera* glucosinolates were degraded to isothiocyanates by

myrosinase (structural formulas in Suppl. Figure 2). Matusheski & Jeffery (2001) defined the breakdown products of glucoraphanin as sulforaphane and sulforaphane nitrile depending on the conditions with a much higher chemoprotective potential of sulforaphane. The hydrolysis product formed from Rhamno-Benzyl-GS was identified as Rhamno-Benzyl-Isothiocyanate (Galuppo et al., 2014). Next to 4-hydroxybenzyl cyanide (lower pH), 4-hydroxybenzyl isothiocyanate was determined as sinalbin hydrolysis products (Galuppo et al., 2014). Interestingly, the authors further found a rapid conversion of 4-hydroxybenzyl isothiocyanate to 4-hydroxybenzyl alcohol in aqueous solution after a few hours. Despite the structural differences between *M. oleifera* glucosinolates and glucoraphanin, the effects on detoxifying enzymes were similar or stronger for the *M. oleifera* glucosinolates. In contrast, no effects were determined for sinalbin, as it is structurally similar to the *M. oleifera* glucosinolates. It can be assumed that differences in gene up-regulation of detoxifying enzymes may be attributed to the instability of 4-hydroxybenzyl isothiocyanate. Furthermore, the additional sugar, rhamnose, in the glucosinolate molecule, can be a possible explanation of the high efficiency of the *M. oleifera* glucosinolates. Our conclusion is supported by Cham (2007), who found that rhamnose is involved as a key molecule in interaction with tumor cells. The author demonstrated that solamargine, a glycoalkaloid with bound sugars (especially rhamnose), showed a high cytotoxicity for cancer cells in the NRU assay. Cham & Daunter (1990) found that the binding of solamargine on tumor cells (murine sarcoma 180) can be mediated through the rhamnose residue. Whereas solasodine (alkaloid without bound sugars) had no effect on the survival rate of cancer cells, solamargine strongly decreased the survival rate (Cham, 2007). Furthermore, the sugar alone had no anticarinogenic potential (Cham, 2007). These findings seem to be analogous to the results found in the present study. Whereas no influence on cell viability (HepG2) was found for 4-hydroxybenzyl glucosinolates (structurally similar to the *M. oleifera* glucosinolates, but without rhamnose) and rhamnose alone (data not presented), the hydrolyzed *M. oleifera* glucosinolates exhibit cytotoxic effects. Due to four OH-groups in the rhamnose molecule, the glycosylated *M. oleifera* glucosinolates are more polar than the corresponding compound without sugar. Consequently, solubility changes. Isothiocyanates show different polarity and water solubility. Whereas sulforaphane is a very polar and hydrophilic molecule, benzyl isothiocyanate (structural similar to the

*M. oleifera* glucosinolates) is poorly soluble in water (Wilson et al., 2011). Therefore, it is conceivable that *M. oleifera* glucosinolates have a higher water solubility, possibly leading to an increased anticancer or chemoprevention property. However, since cancer prevention studies exist for all main glucosinolate structure groups (aliphatic, aromatic, indolic) and their respective breakdown products (isothiocyanate, nitrile, and indole compounds) as reviewed in detail by Watson & Peedy (2010), and on account of the fact that aglycones of flavonoids are generally more bioactive than their glycosylated forms (Xiao, 2015), it is not possible to conclude from glucosinolate structures anything regarding effectiveness.

**Cytotoxicity**

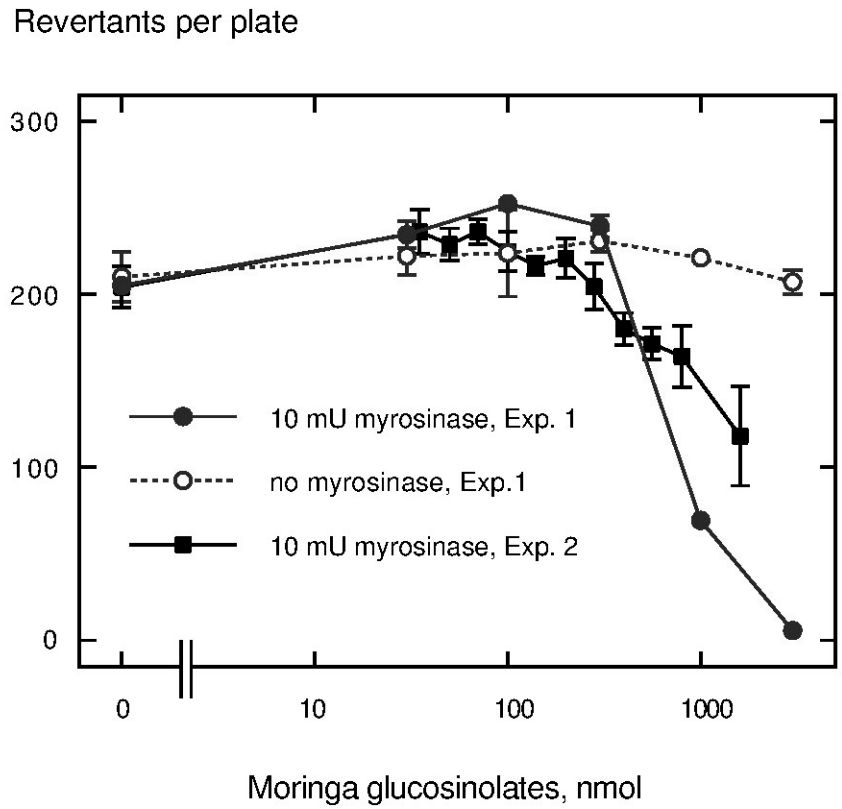
In addition to the induction of detoxifying enzymes and the lack of mutagenic potential of the *M. oleifera* glucosinolates, cytotoxic effects were identified in NRU, SCE, and HPRT assays (Figure 6, Supp. Figure 1, Table 3 and 4). This cytotoxicity of *M. oleifera* glucosinolates was determined in HepG2 cells and V79 cells (for genotoxicity investigations) in our studies. The performed assays do not provide hints for a selective cytotoxicity on the analyzed cells. A putative selectivity for neoplastic cells remains to be analyzed. Furthermore, it remains to be investigated whether cytotoxicity and induction of detoxifying enzymes by *M. oleifera* glucosinolate hydrolysis products can be found *in vivo*. If anticancer effects are to be tested, the time of application, the stage of cancer, and the state of health of the organism, or species (genetic constitution) has to be considered (Haack et al., 2010; Ramadoss et al., 2005).

## Conclusion

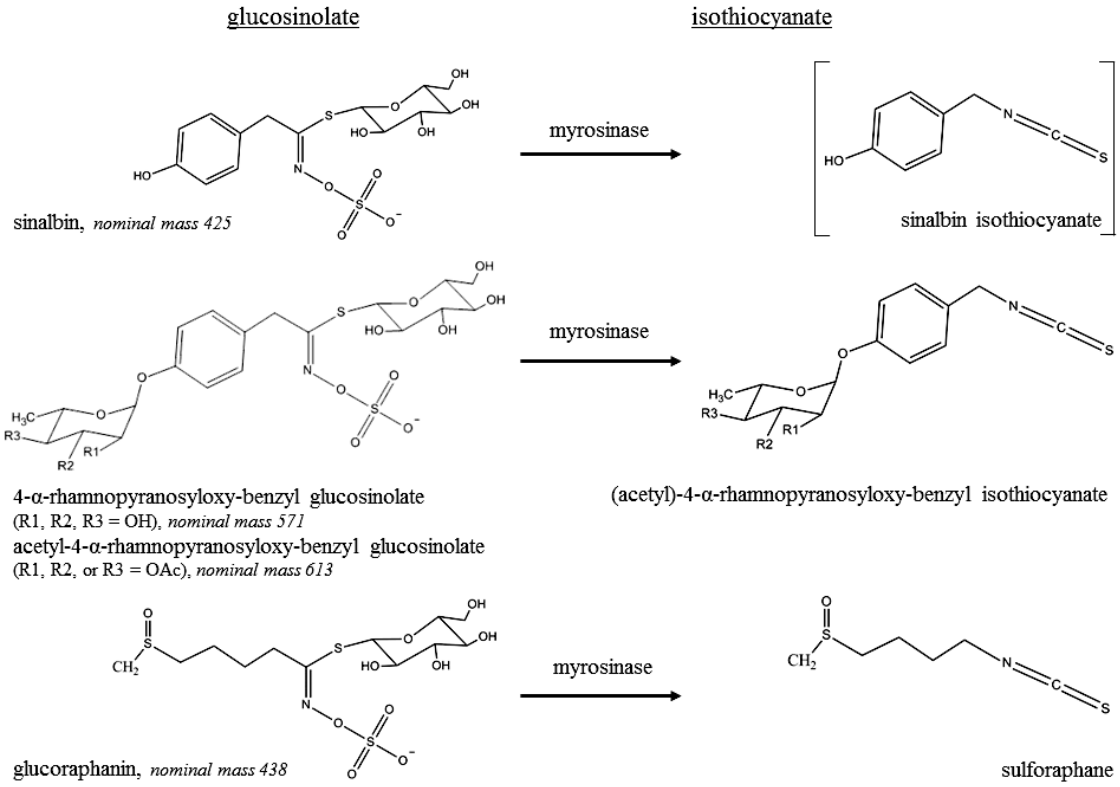
*M. oleifera* appears to contain glucosinolates which exhibit a protective but not mutagenic potential. The two tested glucosinolates of *M. oleifera* induced genes of the phase II and defense system in HepG2 cells through the Nrf2-Keap1-ARE signaling pathway when myrosinase was present. It should be particularly emphasized that detoxifying enzymes were induced with glucosinolate concentrations without toxicity for HepG2 cells. Based on these findings, *M. oleifera* glucosinolates can be added to the group of Nrf2 activators. In conclusion, the health promoting effects of the plant material or substance mixtures (see introduction) described in the literature can be attributed to the breakdown products

of *M. oleifera* glucosinolates. Therefore, the glucosinolates of *M. oleifera* may possess health promoting effects. Further *in vitro* and *in vivo* studies appear to be advisable to examine potential anticarcinogenic effects.

## Supplemented Figures




Suppl. Fig. 1: Mutagenicity test with *Moringa oleifera* glucosinolates in *Salmonella typhimurium* TA104 (mixture of α-4-rhamnopyranosyloxy-benzyl glucosinolate and acetyl-α-4-rhamnopyranosyloxy-benzyl glucosinolate Isomer III was used, molar ratio 4:1)




Suppl. Fig. 2: Chemical structures of sinalbin, the *Moringa oleifera* glucosinolates, and glucoraphanin and their respective isothiocyanates

## References

Arnér, E. S.; Zhong, L. & Holmgren, A. (1999): Preparation and assay of mammalian thioredoxin and thioredoxin reductase. *Methods in Enzymology* 300: 226-239.

Aruna, K. & Sivaramakrishnan, V. M. (1992): Anticarcinogenic effects of some Indian plant products. *Food and Chemical Toxicology* 30: 953-956.

Baasanjav-Gerber, C.; Monien, B. H.; Mewis, I.; Schreiner, M.; Barillari, J.; Iori, R. & Glatt, H. R. (2011a): Identification of glucosinolate congeners able to form DNA adducts and to induce mutations upon activation by myrosinase. *Molecular Nutrition & Food Research* 55: 783-792.

Baasanjav-Gerber, H.; Hollnagel, M.; Brauchmann, J.; Iori, R. & Glatt, H. R. (2011b): Detection of genotoxicants in Brassicales using endogenous DNA as a surrogate target

and adducts determined by 32P-postlabelling as an experimental end point. *Mutagenesis* 26: 407-413.

Banning, A.; Deubel, S.; Kluth, D.; Zhou, Z. & Brigelius-Flohé, R. (2005): The GI-GPx gene is a target for Nrf2. *Molecular and Cellular Biology* 25: 4914-4923.

Bennett, R. N.; Mellon, F. A.; Foidl, N.; Pratt, J. H.; Dupont, M. S.; Perkins L. & Kroon, P. A. (2003): Profiling glucosinolates and phenolics in vegetative and reproductive tissue of the multi-purpose trees *Moringa oleifera* L. (horseradish tree) and Moringa stenopetala L. *Journal of Agricultural and Food Chemistry* 51: 3546-3553.

Bharali, R.; Tabassum, J. & Azad, M.-R. (2003): Chemomodulatory effect *of Moringa oleifera*, Lam, on hepatic carcinogen metabolising enzymes, antioxidant parameters and skin papillomagenesis in mice. *Asian Pacific Journal of Cancer Prevention* 4: 131-139.

Bonnesen, C.; Eggleston, I. M. & Hayes, J. D. (2001): Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. *Cancer Research* 61: 6120-6130.

Bonnesen, C.; Stephensen, P. U.; Andersen, O.; Sorensen, H. & Vang, O. (1999): Modulation of cytochrome P-450 and glutathione S-transferase isoform expression in vivo by intact and degraded indolyl glucosinolate. *Nutrition and Cancer* 33: 178-187.

Brigelius-Flohé, R. & Flohé, L. (2011): Basic principles and emerging concepts in the redox control of transcription factors. *Antioxidants & Redox Signaling* 15: 2335-2381.

Budda, S.; Butryee, C.; Tuntipopipat, S.; Rungsipipat, A.; Wangnaithum, S.; Lee, J.-S. & Kurpradinun, P. (2011): Suppressive effects of *Moringa oleifera* Lam pod against mouse colon carcinogenesis induced by azoxymethane and dextran sodium sulfate. *Asian Pacific Journal of Cancer Prevention* 12: 3221-3228.

Buskov, S.; Hasselstrøm, J.; Olsen, C. E.; Sørensen, H.; Sørensen, J. C. & Sørensen, S. (2000): Supercritical fluid chromatography as a method of analysis for the

determination of 4-hydroxybenzylglucosinolate degradation products. *Journal of Biochemical and Biophysical Methods* 43: 157-174.

Cham, B. E. & Daunter, B. (1990): Solasodine glycosides. Selective cytotoxicity for cancer cells and inhibition of cytotoxicity by rhamnose in mice with sarcoma 180. *Cancer Letters* 55: 221-225.

Cham, B. E. (2007): Solasodine rhamnosyl glycosides specifically bind cancer cell receptors and induce apoptosis and necrosis. Treatment for skin cancer and hope for internal cancers. *Research Journal of Biological Sciences* 2: 503-514.

Clarke, J. D.; Dashwood, R. H. & Ho, E. (2008): Multi-targeted prevention of cancer by sulforaphane. *Cancer Letters* 269: 291-304.

Conaway, C. C.; Jiao, D. & Chung, F. L. (1996): Inhibition of rat liver cytochrome P450 isozymes by isothiocyanates and their conjugates: A structure-activity relationship study. *Carcinogenes*is 17: 2423-2427.

Costa-Lotufo, L. V.; Khan, M. T. H.; Ather, A.; Wilke, D. V.; Jimenez, P. C.; Pessoa, C.; Moraes, M. E. A. de & Moraes, M. O. de (2005): Studies of the anticancer potential of plants used in Bangladeshi folk medicine. *Journal of Ethnopharmacology* 99: 21-30.

Craig, W. J. (1999): Health-promoting properties of common herbs. *The American Journal of Clinical Nutrition* 70suppl: 491S-499S.

Denison, M. S.; Pandini, A.; Nagy, S. R.; Baldwin, E. P. & Bonati, L. (2002): Ligand binding and activation of the Ah receptor. *Chemico-Biological Interactions* 141: 3-24.

Dinkova-Kostova, A.; Holtzdaw, W. D.; Cole, R. N.; Itoh, K.; Wakabayashi, N.; Katoh, Y.; Yamamoto, M. & Talalay, P. (2002): Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences of the United States of America* 99: 11908-11913.

Elbarbry, F. & Elrody, N. (2011): Potential health benefits of sulforaphane: A review of the experimental, clinical and epidemiological evidences and underlying mechanisms. *Journal of Medical Plants Research* 5: 473-484.

Fahey, J. W.; Zhang, Y. & Talalay, P. (1997): Broccoli sprouts: An exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proceedings of the National Academy of Sciences of the United States of America* 94: 10367-10372.

Förster, N.; Ulrichs, C.; Schreiner, M.; Arndt, N.; Schmidt R. & Mewis, I. (2015a): Ecotype variability in growth and secondary metabolite profile in *Moringa oleifera*: Impact of sulfur and water availability. *Journal of Agricultural and Food Chemistry* 63: 2852-2861.

Förster, N.; Ulrichs, C.; Schreiner, M.; Müller, C. T. & Mewis, I. (2015b): Development of a reliable extraction and quantification method for glucosinolates in *Moringa oleifera*. *Food Chemistry* 166: 456-464.

Galuppo, M.; Giacoppo, S.; Nicola, G. R. de; Iori, R.; Navarra, M.; Lombardo, G. E.; Bramanti, P. & Mazzon, E. (2014): Antiinflammatory activity of glucomoringin isothiocyanate in a mouse model of experimental autoimmune encephalomyelitis. *Fitoterapia* 95: 160-174.

Glatt, H. R.; Baasanjav-Gerber, C.; Schumacher, F.; Monien, B. H.; Schreiner, M.; Frank, H.; Seidel, A. & Engst, W. (2011): 1-Methoxy-3-indolylmethyl glucosinolate; a potent genotoxicant in bacterial and mammalian cells: Mechanisms of bioactivation. *Chemico-Biological Interactions* 192: 81-86.

Glatt, H. R.; Piée, A.; Pauly, K.; Steinbrecher, T.; Schrode, R.; Oesch, F. & Seidel, A. (1991): Fjord- and bay- region diol-epoxides investigated on stability, SOS induction in *Escherichia coli*, and mutagenicity in *Salmonella typhimurium* and mammalian cells. *Cancer Research* 51: 1659-1667.

Glatt, H. R.; Schneider, H. & Liu, Y.-G. (2005): V79-hCYP2E1-hSULT1A1, a cell line for the sensitive detection of genotoxic effects induced by carbohydrate pyrolysis products and other food-borne chemicals. *Mutation Research* 580: 41-52.

Gromer, S.; Merkle, H.; Schirmer, R. H. & Becker, K. (2002): Human placenta thioredoxin reductase: preparation and inhibitor studies. *Methods in Enzymology* 347: 382-394.

Haack, M.; Löwinger, M.; Lippmann, D.; Kipp, A.; Pagnotta, E.; Iori, R.; Monien, B. H.; Glatt, H. R.; Brauer, M. N.; Wessjohann, L. A. & Brigelius-Flohé, R. (2010): Breakdown products of neoglucobrassicin inhibit activation of Nrf2 target genes mediated by myrosinase-derived glucoraphanin hydrolysis products. *Biological Chemistry* 391: 1281-1293.

Han, W.; Pentecos, B. T.; Pietropaolo, R. L.; Fasco, M. J. & Spivack, S. D. (2005): Estrogen receptor alpha increases basal and cigarette smoke extract-induced expression of CYP1A1 and CYP1B1, but not GSTP1, in normal human bronchial epithelial cells. *Molecular Carcinogenesis* 44: 202-211.

Hartwell, J. L. (1971): Plants used against cancer: a survey. *Lloydia* 34: 386-425.

Hintze, K. J.; Wald, K. A.; Zeng, H.; Jeffery, E. J. & Finley, J. W. (2003): Thioredoxin reductase in human hepatoma cells is transcriptionally regulated by sulforaphane and other electrophiles via an antioxidant response element. *The Journal of Nutrition* 133: 2721-2727.

Kalthoff, S.; Ehmer, U.; Freiberg, N.; Manns, M. P. & Strassburg, P. (2010): Interaction between oxidative stress sensor Nrf2 and xenobiotic-activated aryl hydrocarbon receptor in the regulation of the human phase II detoxifying UDP-glucoronosyltransferase 1A10. *Journal of Biological Chemistry* 285: 5993-6002.

Kasper, J. W.; Niture, S. K. & Jaiswal, A. K. (2009): Nrf2:INrf2 (Keap1) signaling in oxidative stress. *Free Radical Biology and Medicine* 47: 1304-1309.

Khalafalla, M. M.; Abdellatef, E.; Dafalla, H. M.; Nassrallah, A. A.; Aboul-Enein, K. M.; Lightfoot, D. A.; El-Deeb, F. E. & El-Shemy, H. A. (2010): Active principle from *Moringa oleifera* Lam leaves effective against two leukemias and a hepatocarcinoma. *African Journal of Biotechnology* 9: 8467-8471.

Kim, H.; Jung, Y.; Shin, B. S.; Kim, H.; Song, H.; Bae, S. H.; Rhee, S. G. &. Jeong, W. (2010): Redox regulation of lipopolysaccharide-mediated sulfiredoxin induction, which depends on both AP-1 and Nrf2. *Journal of Biological Chemistry* 285: 34419-34428.

Krajka-Kuzniak, V.; Paluszczak, J.; Szaefer, H. & Baer-Dubowska, W. (2015): The activation of the Nrf2/ARE pathway in HepG2 hepatoma cells by phytochemicals and subsequent modulation of phase II and antioxidant enzyme expression. *Journal of Physiology and Biochemistry* 71: 227-238.

Krajka-Kuzniak, V.; Szaefer, H.; Bartoszek, A. & Baer-Dubowska, W. (2011): Modulation of rat hepatic and kidney phase II enzymes by cabbage juices: Comparison with the effects of indole-3-carbinol and phenyl isothiocyanates. *British Journal of Nutrition* 105: 816-826.

Krehl, S.; Loewinger, M.; Florian, S.; Kipp, A. P.; Banning, A.; Wessjohann, L. A.; Brauer, M. N.; Iori, R.; Esworthy, R. S.; Chu, F.-F. & Brigelius-Flohé, R. (2012): Glutathione peroxidase-2 and selenium decreased inflammation and tumors in a mouse model of inflammation-associated carcinogenesis whereas sulforaphane effects differed with selenium supply. *Carcinogenesis* 33: 620-628.

Kwak, M. K.; Wakabayashi, N. & Kensler, T. W. (2004): Chemoprevention through the Keap1-Nrf2 signaling pathway by phase 2 enzyme inducers. *Mutation Research* 555: 133-148.

Lee, O.; Jain, A. K.; Papusha, V. & Jaiswal, A. K. (2007): An auto-regulatory loop between stress sensors INrf2 and Nrf2 controls their cellular abundance. *Journal of Biological Chemistry* 282: 36412-36420.

Lippmann, D. (2013): Der Einfluss glucosinolatreicher *Brassica* Gemüse auf das endogene Schutzsystem und die entzündungsassoziierte Colonkanzerogenese in der Maus, Dissertation, Universität Potsdam.

Mahmood, K. T.; Mugal, T. & Ul Haq, I. (2010): *Moringa oleifera*: a natural gift - A review. *Journal of Pharmaceutical Sciences and Research* 2: 775-781.

Maron, D. M. & Ames, B. N. (1983): Revised method for the Salmonella mutagenicity test. *Mutation Research* 113: 173-215.

Matés, J. M.; Pérez-Gómez, C. & Núnez de Castro, I. (1999): Antioxidant enzymes and human diseases. *Clinical Biochemistry* 32: 595-603.

Matusheski, N. V. & Jeffery, E. H. (2001): Comparison of the bioactivity of two glucoraphanin hydrolysis products found in broccoli, sulforaphane, and sulforaphane nitrile. *Journal of Agricultural and Food Chemistry* 49: 5743-5749.

Nakajima, M.; Yoshida, R.; Shimada, N.; Yamazaki, H. & Yokoi, T. (2001): Inhibition and inactivation of human cytochrome P450 isoforms by phenethyl isothiocyanate. *Drug Metabolism and Disposition* 29: 1110-1113.

Nguyen, T.; Sherratt, P. J. & Pickett, C. B. (2003): Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annual Review of Pharmacology and Toxicology* 43: 233-260.

Nioi, P. & Hayes, J. D. (2004): Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors. *Mutation Research* 555: 149-171.

Niture, S. K.; Khatri, R. & Jaiswal, A. K. (2014): Regulation of Nrf2 – An update. *Free Radical Biology and Medicine* 66: 36-44.

Parvathy, M. V. S. & Umamaheshwari, A. (2007): Cytotoxic effects of *Moringa oleifera* leaf extracts on human multiple myeloma cell lines. *Trends in Medical Research* 2: 44-50.

Prochaska, H. J. & Santamaria, A. B. (1988): Direct measurement of NAD(P)H:Quinone reductase from cells cultured in microtiter wells: A screening assay for anticarcinogenic enzyme inducers. *Analytical Biochemistry* 169: 328-336.

Rajan, T. S.; Nicola, G. R. de; Iori, R.; Rollin, P.; Bramanti P. & Mazzon, E. (2016): Anticancer activity of glucomoringin isothiocyanate in human malignant astrocytoma cells. *Fitoterapia* 110: 1-7.

Ramadoss, P.; Marcus, C. & Perdew, G. H. (2005): Role of the aryl hydrocarbon receptor in drug metabolism. *Expert Opinion on Drug Metabolism and Toxicology* 1: 1-13.

Repetto, G.; Peso, A. del & Zurita, J. L. (2008): Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature Protocols* 3: 1125-1131.

Saw, C. L.-L.; Clintrón, M.; Wu, T.-Y.; Guo, Y.; Huang, Y.; Jeong, W.-S. & Kong, A.-N. T. (2011): Pharmacodynamics of dietary phytochemical indoles I3C and DIM: Induction of Nrf2-mediated phase II drug metabolizing and antioxidant genes and synergism with isothiocyanates. *Biopharmaceutics & Drug Disposition* 32: 289-300.

Sharma, V. & Paliwal, R. (2014): Potential chemoprevention of 7,12-dimethylbenz[a]anthracene induced renal carcinogenesis by Moringa oleifera pods and its isolated saponin. *Indian Journal of Clinical Biochemistry* 29: 202-209.

Skupinska, K.; Misiewicz-Krzeminska, I.; Stypulkowski, R.; Lubelska, K. & Kasprzycka-Guttman, T. (2009): Sulforaphane and its analogues inhibit CYP1A1 and CYP1A2 activity induced by benzo[a]pyrene. *Journal of Biochemical and Molecular Toxicology* 23: 18-28.

Stewart, B. W. & Wild, C. P. (2014): World Cancer Report 2014, IARC Nonserial Publication, World Health Organization.

Talalay, P. (2000): Chemoprotection against cancer by induction of phase 2 enzymes. *Biofactors* 12: 5-11.

Talalay, P.; Dinkova-Kostova, A. T. & Holtzclaw, W. D. (2003): Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis. *Advances in Enzyme Regulation* 43: 121-134.

Wang, L.; He, X.; Szklarz, G. D.; Bi, Y.; Rojanasakul, Y. & Ma, Q. (2013): The aryl hydrocarbon receptor interacts with nuclear factor erythroid 2-related factor 2 to mediate induction of NAD(P)H:quinoneoxidoreductase 1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Archives of Biochemistry and Biophysics* 537: 31-38.

Watson, R. R. & Preedy, V. R. (2010): Bioactive foods and extracts: Cancer treatment and prevention. Boca Raton, FL: CRC Press, 1. Auflage.

Wilson, E. A.; Ennahar, S.; Zhao, M.; Bergaentzle, M.; Marchioni, E. & Bindler, F. (2011): Simultaneous determination of various isothiocyanates by RP-LC following precolumn derivatization with mercaptoethanol. *Chromatographia* 73(Suppl 1): 137-142.

Xiao, J. (2015): Dietary flavonoid aglycones and their glycosides: which show better biological significance? *Critical Reviews in Food Science and Nutrition*, DOI: 10.1080/10408398.2015.1032400.

Yang, Y. M.; Noh, K.; Han, C. Y. & Kim, S. G. (2010): Transactivation of genes encoding for phase II enzymes and phase III transporters by phytochemical antioxidants. *Molecules* 15: 6332-6348.

Yu, X. & Hensler, T. (2005): Nrf2 as a target for cancer chemoprevention. *Mutation Research* 591: 93-102.

Zhang, Y. & Talalay, P. (1998): Mechanism of differential potencies of isothiocyanates as inducers of anticarcinogenic phase 2 enzymes. *Cancer Research* 58: 4632-4639.

Zhang, Y.; Talalay, P.; Cho, C.-G. & Posner, G. H. (1992): A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure. *Proceedings of the National Academy of Sciences of the United States of America* 89: 2399-2403.

# Diskussion

Obwohl *M. oleifera* ein tropischer/subtropischer, tiefwurzelnder, nicht kälteresistenter Baum ist, konnte ein Bestand an verschiedenen *M. oleifera*-Ökotypen in Tiefbeeten im Gewächshaus in Deutschland etabliert werden. Diese Pflanzen waren die Grundlage für die Durchführung aller in der vorliegenden Dissertation dargestellten Versuche und Analysen. Auch wenn in dieser Arbeit viele Fragestellungen wissenschaftlich bearbeitet und beantwortet werden konnten, bleiben einige Sachverhalte offen und müssten in weiterführenden Untersuchungen analysiert werden. Im Folgenden sollen drei dieser Themenkomplexe diskutiert werden.

**Genetische Diversität verschiedener Ökotypen/Akzessionen von *M. oleifera***

Für die durchgeführten Untersuchungen dieser Arbeit wurden Akzessionen von *M. oleifera* aus den USA (TOT 4880), Thailand (4893, 4951, 4977, 5028, 5077, 7266), Indien (7267), Tansania (7270), sowie aus vom Fachgebiet selbstständig durchgeführten Sammlungen in Taiwan und den Philippinen verwendet, wobei lediglich ausgewählte Vertreter für die Datenerhebungen in den jeweiligen Artikeln einbezogen wurden. Trotzdem diese Herkünfte für die vorliegende Arbeit unter annähernd identischen Bedingungen im Gewächshaus angebaut wurden, zeigten sich Unterschiede hinsichtlich ihres Wachstums und ihres Sekundärmetabolitprofils (Förster et al., 2015a). Auch Patricio et al. (2012) konnten nachweisen, dass sich 18 verschiedene Akzessionen von *M. oleifera* unterschiedlich bei ähnlichen Anbaubedingungen entwickelten. So zeigten die auf den Zentral-Philippinen angebauten Herkünfte, ursprünglich aus Indien, Laos, Philippinen, Taiwan, Tansania, Thailand und den USA stammend, eine unterschiedliche Samenkeimfähigkeit, Pflanzenhöhe, Stammdurchmesser, Triebanzahl, Blattbiomasse und Schotenproduktion. Patricio et al. (2012) konnten auch feststellen, dass 15 der 18 untersuchten Herkünfte einen periodischen wassergesättigten Zustand des Boden überlebten, obwohl *M. oleifera* sehr empfindlich gegenüber Staunässe reagiert und dies bei längerem Anhalten meist eine Wurzelrotte zur Folge hat. Weiterhin beobachteten Abubakar et al. (2011) bei verschiedenen *M. oleifera*-Herkünften aus Nordnigeria morphologische/anatomische Unterschiede des Blattes. Neben Untersuchungen von morphologischen Parametern wie Blattlänge, Fiederblattlänge, Fiederblattbreite und Blattfläche, führten sie auch anatomische Studien durch und bestimmten die

Stomatalänge und –breite der Blattepidermis. Abubakar et al. (2011) stellten Variationen zwischen den untersuchten Herkünften fest, zeigten allerdings auch in einer Clusteranalyse, dass anhand der untersuchten Parameter keine regional spezifischen Gruppierungen vorgenommen werden können. Nach den Autoren deutet das darauf hin, dass zumindest innerhalb von Nordnigeria keine genetische Isolierung der geografischen Herkünfte besteht, der Austausch von genetischen Informationen, ob durch Windbestäubung oder menschliche Übertragung, also stattfindet. Weiterhin stellen die Autoren heraus, dass Variabilitäten in der Blattmorphologie auf evolutionäre Prozesse auf niedrigstem Niveau hindeuten (Abubakar et al., 2011). Palada & Chang (2003) berichteten von einem unterschiedlichen Wachstumsverhalten sowie Blatt-, Blüten- und Schotencharakteristika verschiedener *M. oleifera*-Akzessionen, welche in Taiwan angebaut wurden. Die Autoren empfahlen Anbauern lokal adaptierte Herkünfte zu verwenden, um in Plantagen möglichst einen hohen Ertrag mit gesundem Blattmaterial sowie einer schellen Regeneration nach dem Rückschnitt erzielen zu können (Palada & Chang, 2003). Die Ausprägung von unterschiedlichem Wuchsverhalten sowie eines abweichenden Sekundärmetabolitprofils der *M. oleifera*-Akzessionen unter gleichen Anbaubedingungen deutet auf eine genetische Variabilität hin, welche von den bisher zitierten Autoren jedoch nicht spezifisch untersucht wurde.

Seit dem Jahr 1999 beschäftigten sich verschiedene Autoren verstärkt damit die Diversität von *M. oleifera* mittels DNA basierter Marker zu analysieren. In Untersuchungen zur genetischen Variabilität von *M. oleifera*-Herkünften aus verschiedenen Populationen aus Indien, Malawi und Kenia stellten Muluvi et al. (1999) fest, dass signifikante genetische Unterschiede zwischen den Regionen und Populationen nachgewiesen werden können. Die Autoren führten dies auf einen durch die geografische Isolation eingeschränkten Genfluss zurück. Weitere Faktoren stellen nach Muluvi et al. (1999) die Populationsgröße (korreliert mit genetischer Vielfalt) sowie der herrschende Selektionsdruck dar. Clusteranalysen mittels RAPD-Marker an kultivierten und nichtkultivierten Herkünften von *M. oleifera* von der Küstenregion Tansanias zeigten einen Trend zur genetischen Separation zwischen kultivierten und nichtkultivierten Herkünften auf (Mgendi et al., 2010). Die Autoren führen dies auf die Anforderungen an ihre jeweiligen Umgebungen zurück. Während kultivierte Herkünfte bei optimalen Umweltbedingungen angebaut werden sowie der Schädlingsbefall unter Kontrolle ist, sind die Wildtypen ständig

variierenden Umweltfaktoren unterworfen und müssen sich diesen schnell anpassen (Wettbewerb, natürliche Selektion), um zu überleben. Saini et al. (2013) analysierten genetische Variabilitäten von acht unterschiedlichen in Indien gesammelten, kommerziell angebauten Kultivaren von *M. oleifera*. Es konnte eine große genetische Diversität zwischen den Kultivaren gefunden werden, jedoch ohne ein erkennbares geografisches Muster, was die Autoren auf hohe Genflussraten durch Fremdbestäubung sowie eine weite Verteilung des Pflanzenmaterials zurückführten. Shahzad et al. (2013) bestätigten die Ergebnisse der anderen Autoren, in dem sie 161 *M. oleifera*-Herkünfte aus Asien, Afrika, Nord- und Südamerika sowie von den Karibischen Inseln untersuchten und eine breite genetische Diversität von *M. oleifera*, besonders der aus den Wildsammlungen von Pakistan, einem Ursprungsgebiet von *M. oleifera,* stammenden Herkünfte, feststellten.

Zusammenfassend kann man festhalten, dass die genetische Diversität von *M. oleifera* sehr hoch ist. Abhängig von Verbreitungsmustern, Populationsgröße, geografischer/genetischer Isolierung und Selektionsdruck haben sich Herkünfte (gesammelt als Akzessionen zur Erhaltung in einer Genbank oder anderen Sammlungen) in unterschiedlichem Maße genetisch voneinander separiert. *M. oleifera*-Herkünfte haben sich an unterschiedliche ökologische Lebensbedingungen und damit Umweltansprüche angepasst und können sich daher folglich genetisch sowie physiologisch gering voneinander unterscheiden. Die von uns in dieser Arbeit genutzten *M. oleifera*-Herkünfte (Akzessionen vom AVRDC sowie zwei Wildsammlungen) zeigten unter gleichen Anbaubedingungen hohe Variabilitäten in der Biomasse- und Sekundärmetabolitproduktion. Die in dieser Arbeit genutzten Herkünfte werden daher als *M. oleifera*-Ökotypen bezeichnet.

Die Nutzung verschiedener Ökotypen für einen Anbau auf Standorten mit unterschiedlichen klimatischen oder pedologischen Gegebenheiten scheint ratsam. In der vorliegenden Arbeit konnte gezeigt werden, dass die Ökotypen auf Trockenstress sowie Schwefeldüngung in ganz unterschiedlicher Weise hinsichtlich ihrer Biomasse- und Sekundärmetabolitproduktion reagierten. Die Effekte der Anbauparameter waren stark ökotypabhängig. Zeigen einige Ökotypen z. B. bei wiederholtem, mäßigen Trockenstress im Vergleich zur Kontrollvariante kaum Unterschiede in Biomasse und Glucosinolatgehalt, wiesen andere einen starken Abfall von Biomasse und/oder Anstieg

der Glucosinolatgehalte auf. Schlussfolgernd sollte beim Anbau von *M. oleifera* darauf geachtet werden, dass zum einen Herkünfte/Ökotypen verwendet werden, welche optimal an die Standortverhältnisse angepasst sind. Zusätzlich sollte jedoch, wie von einer Vielzahl an Autoren postuliert (u. a. Mgendi et al., 2010; Saini et al., 2013; Shahzad et al., 2013), der Genpool gesichert werden, um die genetischen Ressourcen von Wild- sowie Kulturformen von *M. oleifera* zu erhalten. Dieser sollte/kann als Basis für zukünftige Selektionen sowie Züchtungsprogramme genutzt werden.

Die in dieser Arbeit gewonnen Erkenntnisse beziehen sich lediglich auf Gewächshausversuche. Es erscheint absolut notwendig das generierte Wissen auf Feldversuche zu transferieren. Hier ist zu empfehlen, dass verschiedenene *M. oleifera*-Ökotypen in ihrem derzeit natürlichen Verbreitungsgebiet sowie fernab von diesem angebaut werden. Parameter, wie Biomasseproduktion, Pflanzenhöhe, Schoten- bzw. Samenproduktion und Glucosinolatgehalt, sollen erhoben werden, um ermitteln zu können wie sich die Ökotypen an die natürlichen Gegebenheiten anpassen können.

### *M. oleifera*-Glucosinolate *in situ* und *in vitro*

Ein Untersuchungsgegenstand dieser Arbeit war es nach einer erfolgreichen Etablierung und Vermehrung von *M. oleifera in vitro* zu testen, ob die Möglichkeit besteht die Glucosinolate dieser Pflanze im Glas anzureichern und folgend zu isolieren. Schon bei ersten Vorversuchen stellte sich heraus, dass das Glucosinolatprofil von *M. oleifera in vitro* ein anderes als *in situ* ist. So setze sich im Kallusgewebe der Glucosinolatgehalt aus Benzylglucosinolat (68,2 %) und Rhamno-Benzyl-GS (30,4 %) zusammen. Ac-Isomer-GS III bildete lediglich 1,4 % des Anteiles. Auf MS-Medium etablierte intakte *M. oleifera*-Pflanzen wiesen zwar immernoch Benzylglucosinolat als Hauptglucosinolat auf (60,6 %), zeigten jedoch auch nachweisbare Anteile aller anderen für diese Pflanze charakteristische Glucosinolate (Rhamno-Benzyl-GS 19,4 %, Ac-Isomer-GS I 2,9 %, Ac-Isomer-GS II 2 %, Ac-Isomer-GS III 12,6 %) sowie *p*-Hydroxybenzylglucosinolat (Sinalbin; 2,5 %). Ein Zusatz von Aktivkohle im Nährmedium und damit Abdunkeln des Wurzelbereiches sorgte für keine Änderung des Glucosinolatprofils. Mit Überführung der *in vitro*-Pflanzen auf sterile Erde nahm der Anteil am Benzylglucosinolat am Gesamtglucosinolatgehalt deutlich ab, der Anteil an Rhamno-Benzyl-GS hingegen zu (Sinalbin 6,3 %, Benzylglucosinolat 34,2 %, Rhamno-Benzyl-GS 41,2 %, Ac-Isomer-

GS I 3,6 %, Ac-Isomer-GS II 2,3 %, Ac-Isomer-GS III 12,3 %). Bennett et al. (2003) und Förster et al. (2015a) detektierten in adulten aus Sämlingen im Gewächshaus oder Freiland aufgezogenen *M. oleifera*-Pflanzen nur Rhamno-Benzyl-GS sowie die drei acetylierten Isomere, jedoch kein Sinalbin oder Benzylglucosinolat. Diese Erkenntnisse legen nahe (wie auch von Graaf et al. (2015) für *Noccaea caerulescens* beobachtet), dass Sinalbin eine Art Präkursor für Rhamno-Benzyl-GS darstellt. Umgekehrt konnten Förster et al. (2015b) feststellen, dass die Isomere sowie das Rhamno-Benzyl-GS schnell hydrolysieren. Außerhalb des pflanzlichen Organismus wurde ein Verlust von zunächst der Acetylgruppe und anschließend der Rhamnose festgestellt. Sinalbin entstand als Folge der Degradation. Auch wenn Förster et al. (2015b) diesen Zerfall nachweisen konnten, gehen Graaf et al. (2015) davon aus, dass die Bindung von Rhamnose an der Seitenkette des Isothiocyanates (Hydrolyseprodukt des Glucosinolates) das Molekül stärker wasserlöslich sowie chemisch stabiler gegenüber dem Isothiocyanat ohne Rhamnose machen. Gesicherte Analyseergebnisse zur Glykosylierung/Stabilität der *M. oleifera*-Glucosinolate bzw. deren Isothiocyanate in Zellen oder lebenden Organismen gibt es allerdings nicht. Für Flavonoide ist die Glykosylierung am besten untersucht, da sie mit einer Vielzahl an medizinischen Effekten (siehe Review von Xiao, 2015) in Verbindung gebracht wird. Vila-Real et al. (2011) geben an, dass multi-glykosylierte Flavonoide schlechter absorbiert werden als mono-glykosylierte Formen oder Aglykone. Xiao (2015) erwähnt in seinem Review, dass eine O-Glykosylierung die Bioaktivität von Flavonoiden generell (Ausnahmen z. B. Anti-HIV-Aktivität, Anti-Allergie-Aktivität) senkt. Aufgrund ihrer Größe und ihrer hohen Polarität gelten glykosylierte Flavonoide als zu hydrophil, um passiv durch biologische Membranen zu diffundieren (Schmitt-Schillig et al., 2005). Weiterhin wiesen glykosylierte Flavonoide eine längere mittlere Verweildauer im Blut auf als ihre Aglykone (Jiang et al., 2008; Review in Xiao, 2015). Křižková et al. (2009) untersuchten den Einfluss der glykosylierten Flavonoide Isoquercetin und Rutin und ihrem Aglykon (Quercetin) auf die Expression von Cytochrom P450 (CYP´s). Es stellte sich heraus, dass Isoquercetin die CYP´s am effektivsten induzierte. Aufgrund der dargelegten und noch vieler weiteren kontroversen Untersuchungsergebnisse diverser Autoren können keine generellen Aussagen hinsichtlich der Absorption, des Stoffwechsels sowie der Verfügbarkeit von Flavonoiden (glykosyliert sowie nicht glykosyliert) gemacht werden (Křižková et al., 2009). Xiao

(2015) nimmt sogar an, dass *in vitro*- und *in vivo*-Untersuchungen zur Bioaktivität unterschiedliche Resultate hervorbringen können.

In Anlehnung an die vielen Untersuchungen mit Flavonoiden kann über die Entstehung von multi-glykosylierten Glucosinolaten in *M. oleifera* nur spekuliert werden. Graaf et al. (2015) geben an, dass die Umwandlung von Sinalbin zu Rhamno-Benzyl-GS durch ein einzelnes Enzym erfolgen kann. Mit einer einzelnen Genmutation und folgendem Funktionsverlust des Enzymes ist es der Pflanze nicht mehr möglich die glykosylierten Formen der Glucosinolate zu synthetisieren (Graaf et al., 2015). Ein Enzym, welches im Zusammenhang mit der Übertragung von Rhamnose auf sekundäre Inhaltsstoffe diskutiert wird, ist die Rhamnosyltransferase. Rhamnosyltransferasen gehören zu den Glykosyltransferasen und katalysieren die Übertragung von Zucker auf andere Moleküle wie z. B. Xenobiotika oder Sekundärmetabolite wie Coumarine oder Flavonoide. Für *Arabidopsis* wurde gezeigt, dass das Enzym UGT78D1 (eine Uridindiphosphat(UDP)-Glykosyltransferase) UDP-Rhamnose als Donor nutzt und den Zucker an die Akzeptoren Kaempferol, Quercetin und Isorhamnetin binden kann (Yonekura-Sakakibara et al., 2008). Nach Roy et al. (2016) findet die Glykosylierung besonders an molekularen Ringstrukturen mit enthaltenen Hydroxylgruppen statt. In *M. oleifera* wurden lediglich aromatische Glucosinolate, also solche mit Benzylring, identifiziert. Der Fakt, dass Sinalbin als Präkursor für die multi-glykosylierten Glucosinolate gilt und die Rhamnose an der Hydroxygruppe des Benzylrings gebunden ist, verstärkt die Vermutung, dass die *M. oleifera*-Glucosinolate unter enzymatischer Einwirkung von Glykosyltransferasen in der Pflanze gebildet werden. Interessanterweise erweist sich die Nutzung von kommerziell erwerbbaren Enzymen wie die Naringinase (Enzymkomplex aus $\alpha$-L-Rhamnosidase und $\beta$-D-Glucosidasen), welche zur Deglykosylierung genutzt wird, als konventionelle chemische Methode zur Produktion von teuren mono-glykosylierten Flavonoiden aus günstigen Ausgangsstoffen und bietet daher ein großes Potential für die die Lebensmittelindustrie sowie Pharmazie (Vila-Real et al., 2011). Die Enzymaktivität kann durch Temperatur und pH-Wert beeinflusst werden (Vila-Real et al., 2011). Die Autoren schlussfolgern anhand ihrer Ergebnisse weiter, dass die Wahl des verwendeten Puffers für die Versuche entscheidend ist, um selektiv Enzymaktivitäten herabzusetzen. Auch in unseren Versuchen konnten wir mit dem Einsatz von Ammoniumacetatpuffer Zerfalls- und Umformungsprozesse der *M. oleifera*-Glucosinolate aufhalten (Förster et

al., 2015b). Auch dies bestärkt die Annahme, dass Enzymaktivitäten für die Bildung sowie den Zerfall der multi-glykosylierten Glucosinolate in *M. oleifera* verantwortlich sind.

**Bioverfügbarkeit der *M. oleifera*-Glucosinolate sowie Übertragbarkeit der biologischen Aktivität auf den Menschen**

In der Pflanze werden Glucosinolate in ihrer glykosylierten stabilen Form in den Vakuolen der meisten Pflanzengewebe oder auch wie bei *Arabidopsis thaliana* in spezifischen Schwefel „S-Zellen“, welche sich zwischen Phloem und Endodermis befinden, gespeichert (Koroleva & Cramer, 2011). Gentrennt von diesen sekundären Pflanzeninhaltsstoffen enthält die Pflanze in den Myrosinzellen der glucosinolatproduzierenden Gewebe die Myrosinase (ß-Thioglucosidase), ein Enzym, welches die intakten Glucosinolate bei Zusammenführung hydrolysieren kann. Myrosinase und Glucosinolate können damit zwar in der gleichen Zelle lokalisiert sein, sind aber durch die Speicherung in verschiedenen Zellkompartimenten voneinander abgegrenzt (Koroleva & Cramer, 2011). Wenn die Zelle zerstört wird, z. B. durch Zerkauen oder Zellverwundung, treffen Glucosinolat und Abbauenzym aufeinander. Nach der Nahrungsaufnahme werden die Glucosinolate partiell intakt durch die Magen-Darm-Schleimhaut absorbiert. Der größte Anteil wird allerdings in den Darmlumen metabolisiert (Barba et al., 2016). Hierbei werden im Dünndarm die Glucosinolate durch die pflanzeneigene Myrosinase über ein instabiles Zwischenprodukt, dem Thiohydroximat-O-Sulfonat, zu verschiedenen Hydrolyseprodukten, wie Isothiocyanaten, Nitrilen, Epithionitrilen, Oxazolidine-2-Thionen oder Indol-3-Carbinolen umgewandelt, welche dann absorbiert werden. Welches dieser Abbauprodukte entsteht, hängt u. a. von der Seitenkettenstruktur des Glucosinolates, von myrosinase-assoziierten (modifizierende Proteine) und myrosinase-bindenden Proteinen (Kuchernig et al., 2012) sowie vom herrschenden pH-Wert und der Eisenverfügbarkeit ab. Nicht hydrolysierte Glucosinolate gelangen in den Dickdarm und werden dort mittels bakterieller Myrosinase hydrolysiert und die Abbauprodukte absorbiert oder ausgeschieden (Barba et al., 2016). Geht die Myrosinaseaktivität z. B. durch Erhitzung verloren, gibt es lediglich die Darmbakterien, welche die Hydrolyse durchführen und die bioaktiven Glucosinolatabbauprodukte entstehen lassen können. Die Glucosinolate

durchqueren aufgrund ihrer Hydrophilie das Verdauungssystem dann intakt bis zum Darm, wo sie von der intestinalen Mikrobiota (Darmflora) hydrolysiert werden (Barba et al., 2016). So konnten Cheng et al. (2004) zeigen, dass *Bifidobacterium*-Stämme der humanen Darmflora Glucosinolate *in vitro* zu Nitrilen metabolisieren können. Luang-In et al. (2015) zeigten, dass ein aus menschlichem Kot isolierter *Escherichia coli*-Stamm das Glucosinolat Sinigrin zum Allyl-Isothiocyanat hydrolysiert. In simulierten Verdauungsversuchen mit Glucoraphanin (unter Abwesenheit von Myrosinase und Darmbakterien) konnte gezeigt werden, dass nur geringe Mengen von intakten Glucosinolaten während des Verdauungsprozesses verloren gehen. So wurden von Maskell & Smithard (1994) 2 – 23 % Verlust im Magen und weitere 7 – 28 % im Dünndarm in *in vitro*-Versuchen ermittelt. Zusammenfassend konnte jedoch gezeigt werden, dass nur ein sehr geringer Teil der intakten Glucosinolate intakt absorbiert werden kann. Für eine effektive Isothiocyanatdosis erscheint daher die pflanzliche Myrosinase essentiell (Angelino et al., 2015), da nur ein sehr geringer Teil der intakten Glucosinolate von spezifischen Darmbakterien hydrolyiert werden kann. So zeigten verschiedene Studien (u. a. Saha et al., 2012; Vermeulen et al., 2008), dass nur ein Bruchteil der Isothiocyanate nach Kochen des Brokkolis (Inaktivierung der Myrosinase) im Vergleich zum Verzehr von Rohmaterial im Blut nachgewiesen werden konnte. Li et al. (2011) schlussfolgerten, dass eine regelmäßige Aufnahme von *Brassica*-Gemüsen das Mikrobiotaprofil im Darm verändert und damit ein höherer Anteil von glucosinolatmetabolisierenden Bakterien vorhanden ist. Die Bioverfügbarkeit von Glucosinolathydrolyseprodukten ist nicht nur von der Glucosinolataufnahme abhängig, sondern auch von ihrer Umwandlung in bioaktive Hydrolyseprodukte bis hin zur Passage durch die Darmwand (Angelino & Jeffery, 2014). Um die Bioverfügbarkeit von Glucosinolaten bewerten zu können, müssen Informationen zur Verdauung, Absorption, der Metabolisierung, der Gewebeverteilung sowie der Bioaktivität eingeholt werden (Deng et al., 2015). Die Bioverfügbarkeit der Glucosinolate hängt nach Holst & Williamson (2004) von folgenden wesentlichen Punkten ab: 1) Vorkommen und Konzentration von Glucosinolaten im Pflanzenmaterial, 2) Hydrolyseprozesse im Pflanzenmaterial während Lagerung und Verarbeitung, 3) Eigenschaften des Pflanzenmaterials, 4) Löslichkeits- sowie physikalisch-chemische Eigenschaften, 4) Stabilität, 5) Ausmaß der Zellzerstörung während des Kauens,

6) Verdauungsprozessen im Magen und Dünndarm und 7) Fermentierungsprozessen im Dickdarm. Die Bioverfügbarkeit der Glucosinolate ist somit u. a. abhängig von der Wasserlöslichkeit, Ionisierbarkeit und Lipophilität. So wird z. B. angegeben, dass die Absorption im Darm bei einem log P-Wert (Wert für Membranpermabilität) zwischen 0,5 – 2 optimal ist (Holst & Williamson, 2004). Dieser hängt jedoch stark von der Seitenkettenstruktur sowie vom Vorhandensein der Sulfatgruppe (Glucosinolat oder Isothiocyanat) ab. So hat Benzylisothiocyanat einen berechneten Log P-Wert von 3,31, Sulforaphan hingegen von 0,72 (Glucoraphanin bei -3,81). Auch wenn die Isothiocyanate der *M. oleifera*-Glucosinolate strukturell sehr ähnlich zum Benzylisothiocyanat sind, liegt die Vermutung nahe, dass der zusätzlich gebundene Zucker am Molekül (Rhamnose +/- Acetylgruppe) die Löslichkeit des Moleküls entscheidend beeinflusst. Nach Graaf et al. (2015) ist das Isothiocyanat durch die Rhamnose stärker wasserlöslich. Ob dies ein Vor- oder Nachteil für die Bioverfügbarkeit der Abbauprodukte der *M. oleifera*-Glucosinolate ist, kann man ohne weiterführende Untersuchungen nicht abschätzen. Holst & Williamson (2004) korrelieren eine bessere Löslichkeit mit einer verringerten Permeabilität. Der Membrantransport muss daher aktiv erfolgen und ist nicht mehr passiv möglich. Allerdings beziehen sich die Autoren bei ihren Angaben auf den Vergleich zwischen intakten Glucosinolaten (wasserlöslich durch gebundenen Glucose) und Isothiocyanaten.

Am besten untersucht ist die Bioverfügbarkeit von Glucoraphanin, einem Glucosinolat aus Brokkoli. Hier wird das intakte Glucosinolat zunächst oxidiert (zu Glucoerysolin) oder reduziert (Glucoerucin) und dann mit Hilfe der Myrosinase in der Leber oder im Darm in die Abbauprodukte Erucin, Erucin-Nitril, Sulforaphanin-Nitril oder Sulforaphan umgewandelt (Angelino & Jeffery, 2014; Saha et al., 2012). Sulforaphan, ein Isothiocyanat von Glucoraphanin, in hohem Maße bioverfügbar ist, da es schnell absorbiert, metabolisiert und wieder ausgeschieden wird. Verschiedene Studien zeigten, dass Sulforaphan über die Darmwand aufgenommen wird, dort mit Glutathion m. H. der Glutathion-S-transferase konjugiert und passiv oder aktiv (Multidrug Resistance-Associated Protein 1) weitertransportiert wird (Traka & Mithen, 2009; Zhang & Callaway, 2002). Der Isothiocyanat-Glutathion-Komplex wird über den Mercaptursäure-Stoffwechselweg weiter metabolisiert und über den Urin ausgeschieden (Traka & Mithen, 2009). Nach 24 h konnte 74 % der aufgenommenen Menge an Glucoraphanin in

Brokkolisprossen als Sulforaphan im Urin nachgewiesen werden (Cramer & Jeffery, 2011). Wird der Isothiocyanat-Glutathion-Komplex jedoch z. B. durch die Glutathion-S-Transferasen wieder aufgelöst, werden die Isothiocyanate frei und können biologisch aktiv werden (Phase I-, Phase II-Metabolismus) (Traka & Mithen, 2009). Die Isothiocyanate von Glucoraphanin sind hoch reaktiv und zeigten in *in vivo* Studien, dass sie über den Nrf2/ARE-Signalweg Phase II- sowie antioxidative Enzyme induzieren können (u. a. Review von Hayes et al., 2008; Traka & Mithen, 2009) oder auch Apoptose in humanen Tumorzellen einleiten können. Analysen von Platz et al. (2016) zeigten, dass auch Glucotropaeolin und dessen Hydrolyseprodukt Benzylisothiocyanat schnell im menschlichen Körper metabolisiert werden. So konnten 1 – 5 h nach oraler Aufnahme von Kapuzinerkresse (*Tropaeolum majus*) maximale Konzentrationen von Benzylisothiocyanat-Metaboliten, wie Benzylisothiocyanat-Cysteinyl-Glycin oder Benzylisothiocyanat-Glutathion, im Blut festgestellt werden (Urin 4 – 6 h). Auch wenn die Glucosinolate von *M. oleifera* ähnliche Effekte wie Glucoraphanin in Zellversuchen *in vitro* zeigten (Förster et al., 2016) und sie strukturell ähnlich zum Benzylglucosinolat sind, lässt sich daraus nicht schlussfolgern wie bioverfügbar sie sind. Dies müsste in weiterführenden Studien untersucht werden. Galuppo et al. (2014) zeigten, dass aus den in den *M. oleifera*-Samen enthaltenen Rhamno-Benzyl-GS durch den enzymatischen Abbau mit Myrosinase bei neutralen Bedingen zu über 99 % Rhamno-Benzylisothiocyanat entsteht. Weiterhin wurde von Faizi et al. (1994) neben dem genannten Isothiocyanat die Entstehung von zwei Nitrilglykosiden (Niazirin und Niazirinin) sowie zwei Thiocarbamate (Niaziminin A und B) beschrieben. Währenddessen die Thiocarbamate sowie das Rhamno-Benzylisothiocyanat blutdrucksendend wirkten, war dieser Effekt für die Nitrile nicht nachweisbar (Faizi et al., 1994). Guevara et al. (1999) zeigten ein antikarzinogenes Potential des Thiocarbamates Niazimicin aus *M. oleifera*. Die Autoren wiesen zum einen aktivitätshemmenden Effekt auf das Epstein-Barr-Virus (EBV-EA) *in vitro* und zum anderen eine tumorhemmende Wirkung im Mausmodell (spätere Bildung von Papillomen auf der Maushaut, verringerte Häufigkeit und Anzahl an Papillomen pro Maus) nach. Es lässt sich daher vermuten, dass das Rhamno-Benzylisothiocyanat sowie die Thiocarbamate die biologisch aktiven Hydrolyseprodukte der Blattglucosinolate von *M. oleifera* sind. Mit der Bestimmung der Log P-Werte dieser Abbauprodukte sowie der

intakten Glucosinolate könnten erste Vergleiche der Bioaktivität zu anderen bereits geprüften Glucosinolaten sowie deren Hydrolyseprodukte angestellt werden.

Viele *in vitro*- sowie *in vivo*-Studien fokussierten sich auf einzelne Glucosinolate. So wurden insbesondere am Beispiel von Glucoraphanin bzw. Sulforaphan Analysen zu antioxidativen, antikarzinogenen oder toxischen Effekten durchgeführt bzw. Umwandlungs- und Abbauprozesse im Tier oder menschlichen Körper untersucht. Auch die Analysen, die in Förster et al. (2016) präsentiert wurden, fokussierten auf die zwei Hauptglucosinolate in den Blättern von *M. oleifera*: Rhamno-Benzyl-GS and Ac-Isomer-GS III. Hier wurden antioxidative sowie antikarzinogene Effekte der isolierten Glucosinolate *in vitro* untersucht. Synergistische sowie antagonistische Effekte sind jedoch in Studien selten festgestellt und gezielt beobachtet worden. In einem großen vom BMBF geförderten Verbundprojekt „Verbesserte Gewinnung und Verarbeitung diätetischer Glucosinolate sowie die Charakterisierung ihrer potenziellen Funktion in der Prävention von Darmkrebs“, wurden u. a. Mäuse mit glucosinolatarmen und –reichen Brokkoli- sowie Pak Choi-Diäten gefüttert. Die Verabreichung der glucosinolatreichen Pak Choi-Diät führte im Vergleich zur glucosinolatarmen zu einer Steigerung der Reportergenaktivität von Nrf2-regulierten Enzymen. Dies könnte die verminderte Colitis, reduzierte Tumorinzidenz sowie Tumoranzahl/Tier im Colon der Versuchstiere erklären (Lippmann, 2013). Lippmann (2013) führt dies auf das in der glucosinolatreichen Pak Choi-Diät in höheren Gehalten enthaltene Gluconapin zurück. Im Gegensatz zu Neoglucobrassicin und Progoitrin induzierte dies in hydrolysierter Form die Reportergenaktivität Nrf2-regulierter Enzyme. Haack et al. (2010) identifizierten antagonistische Effekte von Glucosinolaten. Nach den Autoren verringerten die Hydrolyseprodukte von Neoglucobrassicin die von Glucoraphanin induzierte Promotoraktivität von *NQO1* und *Gpx2*. Folglich sind neben der Pflanzenmatrix an sich, auch die Glucosinolatzusammensetzung sowie –konzentration entscheidende Faktoren für die Regulierung von Nrf2-assoziierten Enzymen. Da sehr viele Faktoren den Verdauungs- und Absorptionsmetabolismus der Glucosinolate und deren Hydrolyseprodukte beeinflussen und damit auch die Ausscheidungswege und Verteilung in Geweben, besteht hier noch sehr viel Forschungsbedarf (Barba et al., 2016). So zeigten z. B. Roland et al. (1996), dass Ballaststoffe die Glucosinolatassimilation modulieren können.

Interessanterweise sind viele auf dem Markt erwerbbare Brokkoli-Nahrungsergänzungsmittel reich an Glucoraphanin, aber enthalten keine Myrosinase. Die Bioverfügbarkeit von Sulforaphan sowie Erucin aus diesen Produkten als bioaktive Substanzen ist sehr gering und könnte nur mit einer externen Zugabe von Myrosinase erhöht werden (Clarke et al., 2011). Auch Fahey et al. (2015) stellten fest, dass bei einer oralen Aufnahme von Brokkolisprossen oder –samen eine Inaktivierung der endogenen Myrosinase eine 3 – 4-fache Verringerung der Bioverfügbarkeit zur Folge hatte. Analysen von ähnlichen Nahrungsergänzungsmitteln von *M. oleifera* an unserem Fachgebiet zeigten sogar, dass in diesen Pulvern keinerlei Glucosinolate nachgewiesen werden können. Vermutlich ist dies auf den Herstellungsprozess der Pulver zurückzuführen. Schon während des Trocknungsprozesses könnten die instabilen Glucosinolate aus den Blättern von *M. oleifera* zerstört und somit im Endprodukt nicht mehr detektiert werden.

## Ausblick

Sehr viele Wissenschaftler arbeiten an diversen Forschungsthemen zu *M. oleifera*. Fragestellungen zum optimalen Anbau, Inhaltsstoffzusammensetzung sowie ernährungsphysologische- und gesundheitsfördernde Effekte von *M. oleifera* auf Mensch und Tier stehen bei diesen Arbeiten im Vordergrund. Auch die vorliegende Arbeit untersucht wertgebende gesundheitsfördernde Inhaltsstoffe in den Blättern von *M. oleifera* sowie deren gezielte Beeinflussung durch Veränderung von Kulturbedingungen. Trotzdem ist es notwendig einige dieser Forschungsschwerpunkte noch detaillierter zu bearbeiten.

Im Zuge dieser Arbeit konnte in Gewächshausversuchen festgestellt werden, dass sich die *M. oleifera*-Akzessionen morphologisch sowie hinsichtlich ihres Inhaltsstoffprofils unter gleichen Anbaubedingungen voneinander unterscheiden. Wir sprechen daher von Ökotypen. Es wurden zwei Ökotypen identifiziert, welche eine starke Wüchsigkeit mit gleichzeitig hohen Gehalten an Glucosinolaten und Flavonoiden aufweisen. Bei diesen zwei Ökotypen war es möglich unter den im Gewächshaus festgelegten Kulturbedingungen viel Blattmaterial mit hohem ernährungsphysiologischen und gesundheitsfördernden Potential mehrfach im Jahr zu ernten. Da sich die Ergebnisse der vorliegenden Analysen lediglich auf die durchgeführten Gewächshausversuche beziehen, ist es ratsam die Ökotypen in Freilandversuchen in den Herkunftsgebieten bzw. in natürlichen Verbreitungsgebieten von *M. oleifera* zu testen. So wird derzeit im Rahmen einer vom DAAD-geförderten Dissertation am Fachgebiet der Urbanen Ökophysiologie der Pflanzen an der Humboldt-Universität zu Berlin untersucht wie sich klimatische sowie pedologische Faktoren in drei agro-ökologische Zonen in Ghana auf das Wachstum sowie das Sekundärmetabolitprofil von zwei *M. oleifera*-Akzessionen auswirken.

Die genetische Diversität von *M. oleifera* ist sehr hoch, da sich die Herkünfte über die Zeit genetisch voneinander separiert haben. Mehrere Forschungsgruppen beschäftigen sich damit Diversitäten von *M. oleifera* mit Hilfe von DNA-basierten Markern zu analysieren. Ziel ist es hier genetische Ressourcen zu sichern und als Grundlage für zukünftige Selektionen sowie Züchtungsprogramme zu nutzen. Zukünftige Genanalysen könnten darauf abzielen *M. oleifera*-Ökotypen zu identifizieren, welche optimal an bestimmte Standorte (Trockentoleranz/-resistenz, Kältetoleranz/-resistenz) angepasst

sind. Umfangreiche Anbauversuche wären damit nicht mehr nötig. Ebenfalls könnte untersucht werden, ob Synthesewegsgene für Sekundärmetabolite in *M. oleifera*, wie für Glucosinolate oder Flavonoide, mittels Marker detektiert werden können. Auch könnte die Aktivität von Genen bestimmt werden. Zum einen wäre es damit möglich das Vorhandensein von spezifischen sekundären Inhaltsstoffen zu prognostizieren und zum anderen könnten Genaktivitäten mit den Inhaltsstoffgehalten in der Pflanze korreliert werden. Eine gezielte Auswahl geeigneter Ökotypen für ein bestimmtes Anbaugebiet, welche auf die jeweiligen Anforderungen angepasst sind, könnte so ohne aufwendige und umfangreiche Anbauversuche getroffen werden.

Obwohl die Glucosinolate in den Blättern von *M. oleifera* bereits identifiziert sind, ist es bis zum jetzigen Zeitpunkt nicht möglich die drei Ac-Isomer-GS den jeweiligen Peaks im HPLC-Chromatogramm zuzuordnen. Wir wissen zwar, dass eines der drei Isomere (in unserem Fall das Ac-Isomer-GS III) das Hauptisomer darstellt, allerdings können wir nicht sagen, an welcher Position die Acetylgruppe bei diesem Isomer an die Rhamnose gebunden ist. Da die Position der Acetylgruppe die Struktur des Glucosinolates verändert, kann nicht ausgeschlossen werden, dass durch die drei Ac-Isomer-GS unterschiedliche bioaktive Effekte vermittelt werden. Zum einen könnte daher in weiterführenden Untersuchungen mittels Kernspinresonanzspektroskopie (NMR-Spektroskopie) aufgeklärt werden, welches der drei Isomere sich hinter den jeweiligen Peaks verbirgt. Weiterhin könnten diese dann fraktioniert und, ähnlich zu den in dieser Arbeit durchgeführten Tests, auf ihr antioxidatives, antikarzinogenes Potential untersucht werden. Es wäre somit möglich Ähnlichkeiten oder Unterschiede der drei Isomere hinsichtlich ihrer Bioaktivität zu identifizieren.

Auch wäre die Anwendung des „Activity Profiling“ eine weitere Möglichkeit um potentielle medizinische Wirksamkeiten der Sekundärmetabolite in *M. oleifera* zu identifizieren. Hierbei wird mit Hilfe eines Computermodells geprüft, ob die Struktur einer chemischen Verbindung in Proteinstrukturen passt (Protein-Ligandeninteraktionsmodelle). Wenn die Funktion des Proteins bekannt ist, kann mit einer Passfähigkeit auf eine potentielle medizinische Wirksamkeit der untersuchten Struktur geschlossen werden. Aktivitäten oder Inaktivitäten von chemischen Verbindungen können mit Hilfe dieser Modelle vorhergesagt werden. Aufbauend

könnten dann gezielt *in vitro*- und später *in vivo*-Versuche angesetzt werden, um die vermuteten Wirksamkeiten explizit in Zellversuchen oder am Lebewesen nachzuweisen.

## Zusammenfassung

*Moringa oleifera* Lam. wird neben der Verwendung der Blätter als Nahrungsmittel besonders in den Tropen und Subtropen, als pflanzliches Arzneimittel vielfältig in der traditionellen und Stammesmedizin genutzt. Auf Basis von Stammeswissen und mündlichen Überlieferungen von Wirksamkeiten wiesen eine Vielzahl an Autoren in wissenschaftlichen Studien u. a. antibiotische, antimikrobielle, antiinflammatorische, antioxidative und antikarzinogene Effekte von *M. oleifera* nach. Ob das antioxidative und/oder antikarzinogene Potential auf die in den Blättern von *M. oleifera* in hohen Konzentrationen vorhandenen multi-glykosylierten Glucosinolate bzw. deren Abbauprodukte zurückgeführt werden kann, war Forschungsgegenstand der vorliegenden Arbeit.

Um Ausgangsmaterial für die Bioaktivitätsanalysen zu generieren, wurden sechs verschiedene *M. oleifera*-Ökotypen im Gewächshaus angebaut. Zwischen den Ökotypen konnten Unterschiede im Sekundärmetabolitprofil (Glucosinolate, Phenolsäuren, Flavonoide) sowie in der Biomasseproduktion festgestellt werden. Zwei Ökotypen, TOT4880 (Herkunft USA) und TOT7267 (Herkunft Indien), zeigten gleichzeitig einen hohen Sekundärmetabolitgehalt sowie Biomassezuwachs und wurden folglich unter den herrschenden Anbaubedingungen im Gewächshaus als „Top-Ökotypen“ identifiziert. Ebenfalls wurde untersucht wie sich Anbaubedingungen, am Beispiel von Trockenheit und Schwefeldüngung, auf das Pflanzenwachstum und die sekundären Inhaltsstoffe auswirken. Auch wenn die Effekte stark vom Ökotyp abhingen, konnte generell gezeigt werden, dass bei Wassermangel die Biomasseakkumulation abnahm, der Glucosinolatgehalt dafür zunahm. Mit Hilfe des Gewächshausanbaus war es auch außerhalb des natürlichen Verbreitungsgebietes von *M. oleifera* möglich ausreichend Blattmasse über die Vegetationsperiode im Jahr zu generieren. Mit Hilfe einer geeigneten Ökotypenauswahl sowie einer gezielten Kontrolle der Anbauparameter (z. B. Bewässerung) zeigten die Blätter hohe Gehalte an Glucosinolaten. Im Gegensatz dazu erwiesen sich *in vitro* etablierte Pflanzen von *M. oleifera* als nicht geeignete Quelle für die Gewinnung von Glucosinolaten. *M. oleifera* konnte zwar unter Verwendung von 0,2 % $HgCl_2$ *in vitro* erfolgreich etabliert und auf MS-Medium mit 0,5 mg/l Benzylaminopurin-Zusatz schnell vermehrt werden, jedoch war das detektierte

Glucosinolatprofil in den Blättern sowie im Kallus im Vergleich zu den im Gewächshaus geernteten Blättern völlig verändert. Das Material konnte daher nicht für die Extraktherstellung genutzt werden. Folglich dienten die geernteten Blätter aus dem Gewächshaus als Grundlage für die Herstellung der Glucosinolatextrakte für diverse Bioaktivitätsuntersuchungen.

Nachdem sich die übliche DIN-Extraktionsweise (Desulfoglucosinolate) als unbrauchbar für die quantitative sowie qualitative Bestimmung der Glucosinolate von *M. oleifera* herausstellte, wurde ein geeignetes Extraktionsverfahren für die Glucosinolate von *M. oleifera* entwickelt. Umformungs- sowie Abbauprozesse der Glucosinolate konnten m. H. der Extraktion der intakten Glucosinolate sowie unter Verwendung von gepufferten Eluenten bei der HPLC-Analytik unterbunden werden. Auch konnte gezeigt werden, dass bei der Herstellung von Standards einzelner *M. oleifera*-Glucosinolate (4-α-Rhamnopyranosyloxy-Benzylglucosinolat, Acetyl-4-α-Rhamnopyranosyloxy-Benzyl-glucosinolat Isomer III) gepufferte Eluenten während des Fraktionierens genutzt werden mussten. Nur so war es möglich stabile Glucosinolatstandards für folgende Untersuchungen in ausreichender Menge bereitzustellen.

Die hergestellten stabilen Glucosinolatstandards (Gesamtextrakt sowie Einzelsubstanzen) wurden verwendet, um potentielle medizinische Wirksamkeiten dieser zu untersuchen. Unter Zugabe von Myrosinase konnten für die *M. oleifera*-Glucosinolate keinerlei genotoxische sowie mutagene Effekte mittels Ames-Test, HPRT- und SCE-Assay ermittelt werden. Reportergenstudien zeigten, dass die Promotoraktivität von Nrf2-Zielgenen, wie *NQO1* und *Gpx2*, durch Glucosinolathydrolyseprodukte von *M. oleifera* bereits bei Glucosinolatkonzentrationen, welche nicht zytotoxisch auf HepG2-Zellen wirkten (1 – 5 µM), signifikant erhöht wurde. Diese Gene des Phase-II- sowie Abwehrsystems in HepG2-Zellen wurden über den Nrf2-Keap1-ARE-Signalweg induziert. Zusammenfassend konnte mittels der durchführten Analysen festgestellt werden, dass die Abbauprodukte der Glucosinolate von *M. oleifera* detoxifizierende sowie antioxidative Effekte vermitteln und folglich ein interessantes Objekt für vertiefende Bioaktivitätsstudien darstellen. Die Ergebnisse werden als Hinweis auf ein antikarzinogenes Potential der Glucosinolate von *M. oleifera* gewertet.

## Referenzen für Einleitung und Diskussion


Abubakar, B. Y.; MuA´zu S., Khan, A. U. & Adamu, A. K. (2011): Morpho-anatomical variation in some accessions of *Moringa oleifera* Lam. from Northern Nigeria. *African Journal of Plant Science* 5: 742-748.

Angelino D. & Jeffery E. (2014): Glucosinolate hydrolysis and bioavailability of resulting isothiocyanates: Focus on glucoraphanin. *Journal of Functional Foods* 7: 67-76.

Angelino D.; Dosz, E. B.; Sun, J.; Hoeflinger, J. L.; Van Tasell, M. L.; Chen, P.; Harnly, J. M.; Miller, M. J. & Jeffery, E. H. (2015): Myrosinase-dependent and –independent formation and control of isothiocyanate products of glucosinolate hydrolysis. *Frontiers in Plant Science* 6: 831-836.

Antwi-Boasiako, C. & Enninful, R. (2011): Effects of growth medium, a hormone, and stem-cutting maturity and length on sprouting in *Moringa oleifera* Lam. *Journal of Horticultural Science & Biotechnology* 86: 619-625.

Anwar, F.; Latif, S.: Ashraf, M. & Gilani, A. H. (2007): *Moringa oleifera*: A food plant with multiple medicinal uses. *Phytotherapy Research* 21: 17-25.

Atawodi, S. E.; Atawodi, J. C.; Idakwo, G. A.; Pfundstein, B.; Haubner, R.; Wurtele, G., Bartsch, H. & Owen, R. W. (2010): Evaluation of the polyphenol content and antioxidant properties of methanol extracts of the leaves, stem, and root barks of *Moringa oleifera* Lam. *Journal of Medicinal Food* 13: 710-716.

Baasanjav-Gerber, C.; Monien, B. H.; Mewis, I.; Schreiner, M.; Barillari, J.; Iori, R. & Glatt, H. R. (2011): Identification of glucosinolate congeners able to form DNA adducts and to induce mutations upon activation by myrosinase. *Molecular Nutrition & Food Research* 55: 783-792.

BAH (Bundesverband der Arzneimittel-Hersteller e. V. (2015): Der Arzneimittelmarkt in Deutschland 2014 – Zahlen und Fakten (https://www.bah-bonn.de/fileadmin/user_upload/BAH-Zahlenbroschuere_2014_final.pdf), (Zugriff 8.5.2017).

Barba, F. J.; Nikmaram, N.; Roohinejad, S.; Khelfa, A.; Zhu, Z. & Koubaa, M. (2016): Bioavailability of glucosinolates and their breakdown products: Impact of processing. *Frontiers in Nutrition* 3: 1-12.

Bellostas, N.; Sorensen, J. C.; Nikiema, A.; Sorensen, H.; Pasternak, D. & Kumar, S. (2010): Glucosinolates in leaves of *Moringa* species grown and disseminated in Niger. *African Journal of Agricultural Research* 5: 1336-1340.

Bennett, R. N.; Mellon, F. A. & Kroon, P. A. (2004): Screening crucifer seeds as sources of specific intact glucosinolates using ion-pair high-performance liquid chromatography negative ion electrospray mass spectrometry. *Journal of Agricultural and Food Chemistry* 52: 428-438.

Bennett, R. N.; Mellon, F. A.; Foidl, N.; Pratt, J. H.; Dupont, M. S.; Perkins, L. & Kroon, P. A. (2003): Profiling glucosinolates and phenolics in vegetative and reproductive tissue of the multi-purpose trees *Moringa oleifera* L. (horseradish tree) and *Moringa stenopetala* L. *Journal of Agricultural and Food Chemistry* 51: 3546-3553.

Bharali, R.; Tabassum, J. & Azad, M. R. (2003): Chemomodulatory effect of *Moringa oleifera*, Lam, on hepatic carcinogen metabolizing enzymes, antioxidant parameters and skin papillomagenesis in mice. *Asian Pacific journal of cancer prevention* 4: 131-139.

Bloem, E.; Haneklaus, S. & Schnug, E. (2007): Comparative effects of sulfur and nitrogen fertilization and post-harvest processing parameters on the glucotropaeolin content of *Tropaeolum majus* L. *Journal of the Science of Food and Agriculture* 87: 1576-1585.

Bloem, E.; Haneklaus, S.; Kleinwächter, M.; Paulsen, J.; Schnug, E. & Selmar, D. (2014): Stress-induced changes of bioactive compounds in *Tropaeolum majus* L. *Industrial Crops and Products* 60: 349-359.

Bouchereau, A.; Clossais-Besnard, N.; Bensaoud, A.; Leport, L. & Renard, M. (1996): Water stress effects on rapeseed quality. *European Journal of Agronomy* 5: 19-30.

Budda, S.; Butryee, C.; Tuntipopipat, S.; Rungsipipat, A.; Wangnaithum, S.; Lee, J.-S. & Kupradinum, P. (2011): Suppressive effects of *Moringa oleifera* Lam pod against mouse colon carcinogenesis induced by azoxymethane and dextran sodium sulfate. *Asian Pacific Journal of Cancer Prevention* 12: 3221-3228.

Cáceres, A.; Cabrera, O.; Morales, O.; Millinedo, P. & Mendia, P. (1991): Pharmacological properties of *Moringa oleifera*. 1: Preliminary screening for antimicrobial activity. *Journal of Ethnopharmacology* 33: 213-216.

Cáceres, A.; Saravia, A.; Rizzo, S.; Zabala, L., De Leon, E. & Nave, F. (1992): Pharmacological properties of *Moringa oleifera*. 1: Screening for antispasmodic, anti-inflammatory and diuretic activity. *Journal of Ethnopharmacology* 36: 233-237.

Cham, B. E. & Daunter, B. (1990): Solasodine glycosides. Selective cytotoxicity for cancer cells and inhibition of cytotoxicity by rhamnose in mice with sarcoma 180. *Cancer Letters* 55: 221-225.

Cham, B. E. (2007): Solasodine rhamnosyl glycosides specifically bind cancer cell receptors and induce apoptosis and necrosis. Treatment for skin cancer and hope for internal cancers. *Research Journal of Biological Sciences* 2: 503-514.

Cheng, D.-L.; Hashimoto, K. & Uda, Y. (2004): *In vitro* digestion of sinigrin and glucotropaeolin by single strains of *Bifidobacterium* and identification of the digestive products. *Food Chemical Toxicology* 42: 351-357.

Chumark, P.; Khunawat, P.; Sanvarinda, Y.; Phornchirasilp, S.; Morales, N. P.; Phivthong-ngam, L.; Ratanachamnong, P.; Srisawat, S. & Pongrapeeporn, S. (2008): The *in vitro* and *ex vivo* antioxidant properties, hypolipidaemic and antiatherosclerotic activities of water extract of *Moringa oleifera* Lam. leaves. *Journal of Ethnopharmacology* 116: 439-446.

Clarke, J. D., Hsu, A., Riedl, K., Bella, D., Schwartz, S. J., Stevens, J. F., & Ho, E. (2011). Bioavailability and inter-conversion of sulforaphane and erucin in human subjects

consuming broccoli sprouts or broccoli supplement in a cross-over study design. *Pharmaceutical Research* 64: 456–463.

Clarke, J. D.; Dashwood, R. H. & Ho, E. (2008): Multi-targeted prevention of cancer by sulforaphane. *Cancer letters* 269: 291-304.

Costa-Lotufo, L. V.; Khan, M. T. H.; Ather, A.; Wilke, D. V.; Jimenez, P. C.; Pessoa, C.; Moraes, M. E. A. de & Moraes, M. O. de (2005): Studies of the anticancer potential of plants used in Bangladeshi folk medicine. *Journal of Ethnopharmacology* 99: 21-30.

Craig, W. J. (1999): Health-promoting properties of common herbs. *The American Journal of Clinical Nutrition* 70 (suppl.): 491S-499S.

Cramer, J. M. & Jeffery, E. H. (2011). Sulforaphane absorption and excretion following ingestion of a semi-purified broccoli powder rich in glucoraphanin and broccoli sprouts in healthy men. *Nutrition and Cancer* 63: 196–201.

Dam, N. M. van; Tytgat, T. O. G. & Kirkegaard, J. A. (2009): Root and shoot glucosinolates: a comparison of their diversity, function and interactions in natural and managed ecosystems. *Phytochemistry Reviews* 8: 171-186.

Decendit, A. & Mérillon, J. M. (1996): Condensed tannin and anthocyanin production in *Vitis vinifera* cell suspension cultures. *Plant Cell Reports* 15: 762-765.

Deng, Q.; Zinoviadou, K. G.; Galanakis, C. M.; Orlien, V.; Grimi, N.; Vorobiev, E.; Lebovka, N. & Barba, F. J. (2015): The effects of conventional a non-conventional processing on glucosinolates and its derived forms, isothiocyanates: Extraction, degradation, and applications. *Food Engineering Reviews* 7: 357-381.

Doerr, B.; Wade, K. L.; Stephenson, K. K.; Reed, S. B. & Fahey, J. W. (2009): Cultivar effects on *Moringa oleifera* glucosinolate content and taste: A pilot study. *Ecology of Food and Nutrition* 48: 100-211.

Eilert, U.; Wolters, B. & Nahrstedt, A. (1981): The antibiotic principle of seeds of *Moringa oleifera* and *Moringa stenopetala*. *Planta Medica* 42: 55-61.

Ezeamuzie, I. C.; Ambakederemo, A. W., Shode, F. O. & Ekwebelem, S. C. (1996): Antiinflammatory effect of *Moringa oleifera* root extract. *International Journal of Pharmacognosy* 34: 207-212.

Fahey, J. W. (2005): *Moringa oleifera*: A Review of the medical evidence for its nutritional, therapeutic, and prophylactic properties. Part 1. *Trees for Life Journal* (http://www.tfljournal.org/article.php/20051201124931586), (Zugriff 8.5.2017).

Fahey, J. W.; Holtzclaw, W. D.; Wehage, S. L.; Wade, K. L.; Stephenson, K. K. & Talalay, P. (2015): Sulforaphane bioavailability from glucoraphanin-rich broccoli: Control by active endogenous myrosinase. *PLOS ONE* 10, 1-13. DOI: 10.1371/journal.pone.0140963.

Faizi, S.; Siddiqui, B. S.; Saleem, R.; Siddiqui, S.; Aftab, K. & Gilani, A. H. (1994): Isolation and structure elucidation of new nitrile and mustard oil glycosides from *Moringa oleifera* and their effect on blood pressure. *Journal of Natural Products* 57: 1256-1261.

Foidl, N.; Makkar, H. P. S. & Becker, K. (2001): The potential of *Moringa oleifera* for agricultural and industrial uses, Konferenz: Development potential for *Moringa* products? (29. Oktober – 2. November 2001, Dar es Salaam, Tanzania).

Förster, N.; Mewis, I.; Glatt, H.R.; Haack, M.; Brigelius-Flohé; Schreiner, M. & Ulrichs, C. (2016): Characteristic single glucosinolates from *Moringa oleifera*: Induction of detoxifying enzymes and lack of genotoxic activity in various model systems. *Food & Function* 7: 4660-4674.

Förster, N.; Ulrichs, C.; Schreiner, M.; Arndt, N.; Schmidt, R. & Mewis, I. (2015a): Ecotype variability in growth and secondary metabolite profile in *Moringa oleifera*: Impact of sulfur and water availability. *Journal of Agricultural and Food Chemistry* 63: 2852-2861.

Förster, N.; Ulrichs, C.; Schreiner, M.; Müller, C. T. & Mewis, I. (2015b): Development of a reliable extraction and quantification method for glucosinolates in *Moringa oleifera*. *Food Chemistry* 166: 456-464.

Fuglie, L. J. & Sreeja, K. V. (2001): Cultivation of *Moringa*, In: Miracle Tree: The Multiple Attributes of *Moringa*; Fuglie, I. J., Ed.; CTA and CWS: Dakar, Senegal, 153-158.

Fuglie, L. J. (2001): Combating malnutrition with *Moringa*, Konferenz: Development potential for *Moringa* products? (29. Oktober – 2. November 2001, Dar es Salaam, Tanzania).

Galuppo, M.; Giacoppo, S.; Nicola, G. R. de; Iori, R.; Navarra, M.; Lombardo, G. E.; Bramanti, P. & Mazzon, E. (2014): Antiinflammatory activity of glucomoringin isothiocyanate in a mouse model of experimental autoimmune encephalomyelitis. *Filoterapia* 95: 160-174.

Glatt H.; Baasanjav-Gerber, C.; Schumacher, F.; Monien, B. H.; Schreiner, M.; Frank, H.; Seidel, A. & Engst, W. (2011): 1-Methoxy-3-indolylmethyl glucosinolate; a potent genotoxicant in bacterial and mammalian cells: Mechanisms of bioactivation. *Chemico-Biological Interactions* 192: 81-86.

Graaf, R. M. de; Krosse, S.; Swolfs, A. E. M.; Brinke, E. te; Prill, N.; Leimu, R.; van Galen, P. M.; Wang, Y.;Aarts, M. G. M. & von Dam, N. M. (2015): Isolation and identification of 4-α-rhamnosyloxy benzyl glucosinolate in *Noccaea caerulescens* showing intraspecific variation. *Phytochemistry* 110: 166-171.

Guevara, A. P.; Vargas, C.; Sakurai, H.; Fujiwara, Y.; Hshimoto, K.; Maoka, T.; Kozuka, M; Ito, Y.; Tokuda, H. & Nishino, H. (1999): An antitumor promoter from *Moringa oleifera* Lam. *Mutation Research* 440: 181-188.

Haack, M.; Löwinger, M.; Lippmann, D.; Kipp, A.; Pagnotta, E.; Iori, R.; Monien, B. H.; Glatt, H.; Brauer, M. N.; Wessjohann, L. A. & Brigelius-Flohé, R. (2010): Breakdown products of neoglucobrassicin inhibit activation of Nrf2 target genes mediated by myrosinase-derived glucoraphanin hydrolysis products. *Biological Chemistry* 391: 1281-1293.

Hayes, J. D.; Kelleher, M. O. & Eggleston, I. M. (2008): The cancer chemopreventive actions of phytochemicals derived from glucosinolates. *European Journal of Nutrition* 47 (Suppl 2): 73-88.

Holst, B., & Williamson, G. (2004). A critical review of the bioavailability of glucosinolates and related compounds. *Natural Product Reports* 21: 425–447.

Iqbal, M. A.; Cheema, Z. A. & Afzal, M. I. (2015): Evaluation of forage soybean (*Glycine max* L.) germination and seedling growth enhancement by seed priming techniques. *American-Eurasian Journal of Agricultural & Environmental Sciences* 15: 1198-1203.

Jiang, J.; Yuan, S.; Ding, J.; Zhu, S.; Xu, H.; Chen, T.; Cong, X.; Xu, W. & Dai, Y. (2008): Conversion of puerarin into its 7-*O*-glycoside derivates by *Microbacterium oxydans* (CGMCC 1788) to improve water solubility and pharmacogenetic properties. *Applied Microbiology and Biotechnology* 81: 647-657.

Khalafalla, M. M.; Abdellatef, E.; Dafalla, H. M.; Nassrallah, A. A.; Aboul-Enein, K. M.; Lightfoot, D. A.; El-Deeb, F. E. & El-Shemy, H. A. (2010): Active principle from *Moringa oleifera* Lam. leaves effective against two leukemias and a herpatocarcinoma. *African Journal of Biotechnology* 9: 8467-8471.

Kholif, A. E.; Gouda, G. A.; Morsy, T. A.; Salem, A. Z. M.; Lopez, S. & Kholif, A. M. (2015): *Moringa oleifera* leaf meal as a protein source in lactating goat´s diets: Feed intake, digestibility, ruminal fermentation, milk yield and composition, and its fatty acids profile. *Small Ruminant Research* 129: 129-137.

Kirsh, V. A.; Peters, U.; Mayne, S. T.; Subar, A. F.; Chatterjee, N.; Johnson, C. C.; Hayes, R. B. (2007): Prospective study of fruit and vegetable intake and risk of prostate cancer. *Journal of the National Cancer Institute* 99: 1200-1209.

Kopsell, D. A.; Barickmann, T. C.; Sams, C. E. & McElroy, J. S. (2007): Influence of nitrogen and sulfur on biomass production and carotenoid and glucosinolate concentration in watercress (*Nasturtium officinale* R. Br.). *Journal of Agricultural and Food Chemistry* 55: 10628-10634.

Koroleva, O. A. & Cramer, R. (2011): Single-cell proteomic analysis of glucosinolate-rich S-cells in *Arabidopsis thaliana*. *Methods* 54: 413-423.

Krajka-Kuzniak, V.; Paluszczak, J.; Szaefer, H. & Baer-Dubowska, W. (2015): The activation of the Nrf2/ARE pathway in HepG2 hepatoma cells by phytochemicals and subsequent modulation of phase II and antioxidant enzyme expression. *Journal of Physiology and Biochemistry* 71: 227-238.

Křižková, J.; Burdová, K.; Stiborová, M; Křen, V. & Hodek P. (2009): The effects of selected flavonoids on cytochromes P450 in rat liver and small intestine. *Interdisciplinary Toxicology* 2: 201-204.

Kuchernig, J. C.; Burow, M. & Wittstock, U. (2012): Evolution of specifier proteins in glucosinolate-containing plants. *BMC Evolutionary Biology* 12: 127–141.

Kumar, V.; Pandey, N.; Mohan, N. & Singh, R. P. (2012): Antibacterial & antioxidant activity of different extract of *Moringa oleifera* leaves – an *in-vitro* study. *International Journal of Pharmaceutical Sciences Review and Research* 12: 89-94.

Li, F.; Hullar, M. A.; Beresford, S. A. & Lampe, J. W. (2011): Variation of glucoraphanin metabolism *in vivo* and *ex vivo* by human gut bacteria. *British Journal of Nutrition* 106: 408–416.

Lippmann, D. (2013): Der Einfluss glucosinolatreicher *Brassica* Gemüse auf das endogene schutzsystem und die entzündungsassoziierte Colonkanzerogenese in der Maus. Dissertation, Universität Potsdam.

Luang-In, V.; Narbad, A.; Cebeci, F.; Bennett, M. & Rossiter, J. T. (2015): Identification of proteins possibly involved in glucosinolate metabolism in *L. agilis* R16 and *E. coli* VL8. *The Protein Journal* 34: 135–146.

Mahmood, K. T.; Mugal, T. & Ul Haq, I. (2010): *Moringa oleifera*: a natural gift – A review. *Journal of Pharmaceutical Sciences and Research* 2: 775-781.

Mangale Sapana, M.; Chonde Sonal, G. & Raut, P. D. (2012): Use of *Moringa oleifera* (drumstick) seed as natural absorbent and an antimicrobial agent for ground water treatment. *Research Journal of Recent Sciences* 1: 31-40.

Maskell, I & Smithard, R. (1994): Degradation of glucosinolates during *in vitro* incubations of rapeseed meal with myrosinase (EC 3.2.3.1) and with pepsin (EC 3.4.23.1)-hydrochloric acid, and contents of porcine small intestine and caecum. *British Journal of Nutrition* 72: 455–466.

Mehta, J.; Shukla, A.; Bukhariya, V. & Charde, R. (2011): The magic remedy of *Moringa oleifera*: An overview. *International Journal of Biomedical and Advance Research* 2: 215-227.

Meyer, H. J. & Van Staden, J. (1995): The in vitro production of an anthocyanin from callus cultures of *Oxalis linearis*. *Plant Cell, Tissue and Organ Culture* 40: 55-58.

Mgendi, M. G.; Manoko, M. K. & Nyomora, A. M. (2010): Genetic diversity between cultivated and non-cultivated *Moringa oleifera* Lam. provenances assessed by RAPD markers. *Journal of Cell and Molecular Biology* 8: 95-102.

Muluvi, G. M.; Sprent, J. I.; Soranzo, N.; Provan, J.; Odee, D.; Folkard, G.; McNicol, J. W. & Powell, W. (1999): Amplified fragment length polymorphism (AFLP) analysis of genetic variation in *Moringa oleifera* Lam. *Molecular Ecology* 8: 463-470.

Murakami, A.; Kitazono, Y.; Jiwajinda, S.; Koshimizu, K. & Ohigashi, H. (1998) Niaziminin, a thiocarbamate from the leaves of *Moringa oleifera*, holds a strict structural requirement for inhibition of tumor-promoter-induced Epstein-Barr virus activation. *Planta Medica* 64: 319-323.

Nikkon, F.; Saud, Z. A.; Rahman, M. H. & Haque, M. E. (2003): *In vitro* antimicrobial activity of the compound isolated from chloroform extract of *Moringa oleifera* Lam. *Pakistan Journal of Biological Sciences* 6: 1888-1890.

Palada, M. C. & Chang, L. C. (2003): Suggested cultural practices for *Moringa*. International cooperators´ guide, AVRDC pub # 03-545.

Parvathy, M. V. S. & Umamaheshwari, A. (2007): Cytotoxic effect of *Moringa oleifera* leaf extracts on human multiple myeloma cell lines. *Trends in Medical Research* 2: 44-50.

Patricio, H. G.; Palada, M. C. & Ebert A. W. (2012): Adaptability and horticultural characterization of *Moringa* accessions under Central Philippines conditions. Konferenz: High value vegetables in Southeast Asia: Production, Supply and Demand, *Proceedings Seaveg 2012*, (Januar 2012, Chiang Mai, Thailand).

Platz, S.; Kühn, C.; Schiess, S.; Schreiner, M.; Kemper, M.; Pivovarova, O.; Pfeiffer, A. F. H. & Rohn, S. (2016): Bioavailability and metabolism of benzyl glucosinolate in humans consuming Indian cress (*Tropaeolum majus* L.). *Molecular Nutrition & Food Research* 60: 652-660.

Price, M. L. (2007): The *Moringa* Tree. ECHO Technical Note (http://chenetwork.org/files_pdf/Moringa.pdf), (Zugriff 8.5.2017).

Ramachandran, C.; Peter, K. V. & Gopalakrishnan, P. K. (1980): Drumstick (*Moringa oleifera*): A multipurpose Indian vegetable. *Economic Botany* 34: 276-283.

RKI & GEKID (2015): Krebs in Deutschland 2011/2012. 10. Ausgabe. Robert Koch-Institut (Hrsg.) und die Gesellschaft der epidemiologischen Krebsregister in Deutschland e.V. (Hrsg.), Berlin (http://www.gekid.de/Doc/krebs_in_deutschland_2015.pdf), (Zugriff 8.5.2017).

Roland, N.; Rabot, S. & Nugon-Baudon L. (1996): Modulation of the biological effects of glucosinolates by inulin and oat fibre in gnotobiotic rats inoculated with a human whole faecal flora. *Food Chemical Toxicology* 34: 671–677.

Roy, J. le; Huss, B.; Creach, A.; Hawkins, S.; Neutelings, G. (2016): Glycosylation is a major regulator of phenylpropanoid availability and biological activity in plants. *Frontiers in Plant Science*, DOI: 10.3389/fpls.2016.00735.

Saha, S.; Hollands, W.; Teucher, B.; Needs, P .W.; Narbad, A.; Ortori, C. A.; Barrett, D. A.; Rossiter, J. T.; Mithen, R. F. & Kroon, P. A. (2012): Isothiocyanate concentrations and interconversion of sulforaphane to erucin in human subjects

after consumption of commercial frozen broccoli compared to fresh broccoli. *Molecular Nutrition and Food Research* 56: 1906–1916.

Saini, R. K.; Saad, K. R.; Ravishankar, G. A.; Giridhar, P. & Shetty, N. P. (2013): Genetic diversity of commercially grown *Moringa oleifera* Lam. cultivars from India by RAPD, ISSR and cytocrhom $P_{450}$-based markers. *Plant Systematics and Evolution* 299: 1205-1213.

Saw, C. L.-L.; Clintrón, M.; Wu, T.-Y.; Guo, Y.; Huang, Y.; Jeong, W.-S. & Kong, A-N. T. (2011): Pharmacodynamics of dietary phytochemical indoles I3C and DIM: Induction of Nrf2-mediated phase II drug metabolizing and antioxidant genes and synergism with isothiocyanates. *Biopharmaceutics & Drug Disposition* 32: 289-300.

Schmitt-Schillig, S.; Schaffer, S.; Weber, C. C.; Eckert, G. P. & Müller. W. E. (2005): Flavonoids and the aging brain. *Journal of Physiology and Pharmacology* 56 Suppl. 1: 23-36.

Shahzad, U.; Khan, M. A.; Jaskani, M. J.; Khan, I. A. & Korban, S. S. (2013): Genetic diversity and population structure of *Moringa oleifera*. *Conservation Genetics* 14: 1161-1172.

Sharma, V. & Paliwal, R. (2014): Potential chemoprevention of 7,12-dimethylbenz[a]anthracene induced renal carcinogenesis by *Moringa oleifera* pods and its isolated saponin. *Indian Journal of Clinical Biochemistry* 29: 202-209.

Siddhuraju, P. & Becker, K. (2003): Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringa oleifera* Lam.) leaves. *Journal of Agricultural and Food Chemistry* 51: 2144-2155.

Sreelatha, S.; Jeyachitra, A. & Padma, P.R. (2011): Antiproliferation and induction of apoptosis by *Moringa oleifera* leaf extract on human cancer cells. *Food and Chemical Toxicology* 49: 1270-1275.

Srikanth, V. S.; Mangala, S. & Subrahmanyam, G. (2014): Improvement of protein energy malnutrition by nutritional intervention with *Moringa oleifera* among Anganwadi children in rural area in Bangalore, India. *International Journal of Scientific Study* 2: 32-35.

Stewart, B. W. & Wild, C. P. (2014): World Cancer Report 2014, IARC Nonserial Publication, World Health Organisation.

Sultana, N.; Alimon, A. R.; Huque, K. S.; Sazili, A. Q.; Yaakub, H.; Hossain, J. & Baba, M. (2015): The feeding value of *Moringa* (*Moringa oleifera*) foliage as replacement to conventional concentrate diet in Bengal goats. *Advances in Animal and Veterinary Sciences* 3: 164-173.

Thurber, M. D. & Fahey J. W. (2009): Adoption of *Moringa oleifera* to combat under-nutrition viewed through the lens of the „Diffusion of Innovations" Theory. *Ecology of Food and Nutrition* 48: 212-225.

Torre, L. A.; Bray, F.; Siegel, R. L.; Ferlay, J.; Lortet-Tieulent, J. & Jemal, A. (2015): Global Cancer Statistics, 2012. *A Cancer Journal for Clinicians* 65: 87-108.

Traka, M. & Mithen, R. (2009): Glucosinolates, isothiocyanates and humen health. *Phytochemistry Reviews* 8: 269-282.

Verhoeven, D. T. H.; Goldbohm, R. A.; van Poppel, G.; Verhagen, H. & van den Brandt, P. A. (1996): Epidemiological studies on Brassica vegetables and cancer risk. *Cancer Epidemiology, Biomarkers & Prevention* 5: 733-748.

Verma, A. R.; Vijayakumar, M.; Mathela, C. S. & Rao, C. V. (2009): *In vitro* and *in vivo* antioxidant properties of different fractions on *Moringa oleifera* leaves. *Food and Chemical Toxicology* 47: 2196-2201.

Vermeulen, M.; Klöpping-Ketelaars, I. W. A. A.; Berg, R. v. d. & Vaes, W. H. J. (2008): Bioavailability and kinetics of sulforaphane in humans after consumption of cooked versus raw broccoli. *Journal of Agricultural and Food Chemistry* 56: 10505-10509.

Vila-Real, H.; Alfaia, A. J.; Bronze, M. R.; Calado, A. R. R. & Ribeiro, M. H. L. (2011): Enzymatic synthesis of the flavones glucosides, pruning and isoquercetin, and the aglycones naringenin and quercetin, with selective $\alpha$-L-rhamnosidase and $\beta$-D-glucosidase activities of naringinase. *Enzyme Research*, DOI: 10.4061/2011/692616.

Wielanek, M. & Urbanek, H. (1999): Glucotropaeolin and myrosinase production in hairy root cultures of *Tropaeolum majus*. *Plant Cell, Tissue and Organ Culture* 57: 39-45.

Xiao, D.; Vogel, V. & Singh, S. V. (2006): Benzyl isothiocyanate – induced apoptosis in human breast cancer cells is initiated by reactive oxygen species and regulated by Bax and Bak. *Molecular Cancer Therapeutics* 5: 2931-2945.

Xiao, J. (2015): Dietary flavonoid aglycones and their glycosides: Which show better biological significance? *Critical Reviews in Food Science and Nutrition*, DOI: 10.1080/10408398.2015.1032400.

Yang, R.-Y.; Chang, L.-C.; Hsu, J.-C.; Weng, B. B. C.; Palada, M. C.; Chadha, M. L. & Levasseur, V. (2006): Nutritional and functional properties of *Moringa* leaves – from germplasm, to plant, to food, to health, Konferenz: *Moringa* and other highly nutritious plant resources: Strategies, standards and markets for a better impact on nutrition in Africa (16. – 18. November 2006, Accra, Ghana, Africa).

Yasmeen, A.; Basra, S. M. A.; Ahmad, R. & Wahid, A. (2012): Performance of late sown wheat in response to foliar application of *Moringa oleifera* Lam. leaf extracts. *Chilean Journal of Agricultural Research* 72: 92-97.

Yonekura-Sakakibara, K.; Tohge, T.; Matsuda, F.; Nakabayashi, R.; Takayama, H.; Niida, R.; Watanabe-Takahashi, A.; Inoue, E. & Saito, K. (2008): Comprehensive flavonol profiling and transcriptome coexpression analysis leading to decoding gene-metabolite correlations in *Arabidopsis*. *The Plant Cell* 20: 2160-2176.

Zhang, H.; Schonhof, I.; Krumbein, A.; Gutezeit, B.; Li, L.; Stützel, H. & Schreiner, M. (2008): Water supply and growing season influence glucosinolate concentration

and composition in turnip root (*Brassica rapa* ssp. *rapifera* L.). *Journal of Plant Nutrition and Soil Science* 171: 255-265

Zhang, Y. & Callaway, E. C. (2002): High cellular accumulation of sulphoraphane, a dietary anticarcinogen, is followed by rapid transporter-mediated export as a glutathione conjugate. *Biochemical Journal* 364: 301-307.

Zhang, Y. & Talalay, P. (1998): Mechanism of differential potencies of isothiocyanates as inducers of anticarcinogenic phase 2 enzymes. *Cancer Research* 58: 4632-4639.

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