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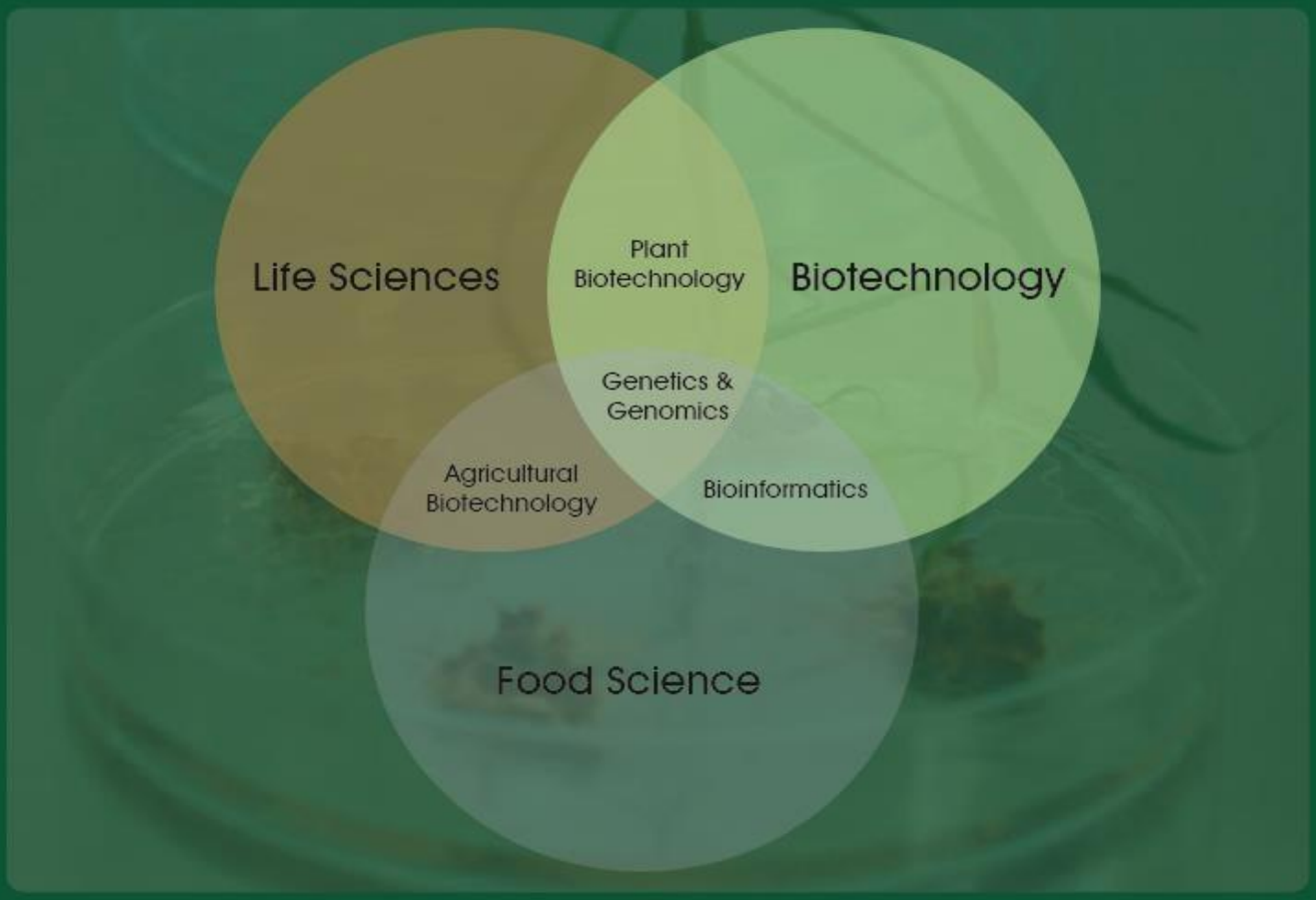

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10.12. 2019
Editör
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From The Editor;

Dear Readers and Authors,

As “International Journal of Life Sciences and Biotechnology”, we are pleased and honored to present the fifth issue of the journal. "International Journal of Life Sciences and Biotechnology" is an international double peer-reviewed open access academic journal published on the basis of research-development and code of practice.

The aims of this journal are to contribute in theoretical and practical applications in relevant researchers of Life Sciences, Biology, Biotechnology, Bioengineering, Agricultural Sciences, Food Biotechnology and Genetics institutions and organizations in Turkey, and to publish solution based papers depending on the principle of impartiality and scientific ethics principles, focusing on innovative and added value work, discussing the current and future.

With these thoughts, We are especially thankful to academicians honoring with the articles, valuable scientists involved in editorial boards and reviewers for their contributions to the evaluation processes with through their opinions/ideas/contributions/criticisms in fifth issue of "International Journal of Life Sciences and Biotechnology". Hope to see you in the next issue…

10.12.2019
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Bolu ve Tokat İllerindeki Buğday Sap Atıklarının Enerji Potansiyel Değerlerinin Karşılaştırmalı Teorik Analizi

Muhammed Tasova[1*] Ismail Naneli[2]

ÖZET

Biyogaz enerjisi, artan küresel enerji talebinin karşılanması için atılan adımlardan olumlu etkilenmektedir. Bu sebeple ülkemizin sahip olduğu organik madde potansiyelinin fazla olması bu atıkların enerjiye dönüştürülme çalışmalarını da hızlandırmıştır. Bu çalışmada, Bolu ve Tokat illerinde bulunan buğday sap atıklarının biyokütle, biyogaz ve enerji potansiyel değerleri kıyaslanmıştır. Bolu ve Tokat illerine ait atık ve kuru madde potansiyelleri sırasıyla, 2827.03-6187.41 ton ve 2459.51-5444.92 ton olarak tespit edilmiştir. İlave olarak, elde edilebilecek uçucu kuru madde potansiyelinin 1345.76-5383.04 ton ve metan potansiyelinin 614.88-2459.519 CH_4 kg olduğu belirlenmiştir. Enerji potansiyellerinin ise 22135.61-48447.39 MJ olarak bulunmuştur. Bolu ilindeki buğday sap atıklarından elde edilebilecek enerji potansiyelinin Tokat iline oranının ortalama % 45.69 daha fazla olduğu tespit edilmiştir.

MAKALE GEÇMİŞİ
Geliş
29 Mayıs 2019
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21 Temmuz 2019

ANAHTAR KELİMELER
Buğday sapı, kuru madde, enerji potansiyeli, Bolu, Tokat

A Comparative Theoretical Analysis of the Energy Potential Values of Wheat Straw Waste in Bolu and Tokat Provinces

ABSTRACT

Biogas energy is positively affected by the steps taken to meet the growing global energy demand. For this reason, the potential of the organic matter of our country is high and the efforts to transform these wastes into energy have also gained momentum. In this study, biomass, biogas and energy potential values of wheat straw wastes in Bolu and Tokat provinces were compared. Wet waste weight and dry matter potentials of Bolu and Tokat provinces were determined as 2827.03-6187.41 tons and 2459.51-5444.92 tons respectively. However, the potential for volatile dry matter obtained was found to be 1345.76-5383.04 tons and the methane potential was 614.88-2459.519 CH_4 kg. Energy potentials were found to be 22135.61-48447.39 MJ. In Bolu province, the ratio of energy potential obtained from wheat straw wastes found to be 45.69% higher than Tokat province.

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Wheat straw, dry matter, energy potential, Bolu, Tokat

Giriş

Nüfus artışıyla birlikte enerji tüketim değerlerinin de aynı doğrultuda artış gösterdiği bilinmektedir. Günümüzde enerji üretiminde ham madde kaynağı olarak yoğunlukta fosil

[1]Tokat Gaziosmanpaşa Üniversitesi Ziraat Fakültesi Biyosistem Mühendisliği Bölümü/Tokat
[2]Tokat Gaziosmanpaşa Üniversitesi Ziraat Fakültesi Tarla Bitkileri Bölümü/Tokat
*Sorumlu yazar: muhammed.tasova@gop.edu.tr

kökenli (kömür, doğalgaz, petrol) yakıtlar kullanılmaktadır. Rezerv ile tüketim miktarları kıyaslandığında fosil yakıtların hızla tükendiği ve gelecekte enerji ihtiyacını karşılayamayacağı belirtilmektedir. Bu sebeple fosil yakıtlara alternatif olarak kullanılan yenilenebilir enerji kaynaklarına yapılan yatırımların arttırılması ve üretim kaynaklarının daha etkin kullanılmasıyla bu durumun aşılacağı ön görülmektedir [1]. Yenilenebilir enerji kaynakları arasında yer alan biyokütle enerjisi hızlı bir şekilde gelişmekte ve yaygınlaşmaktadır. Biyokütle enerjileri (biyogaz, biyodizel, biyoalkol) içerisinde de biyogazın popülerliği her geçen gün artmaktadır.

Biyogaz, organik kökenli atıkların fermantatif ortamda farklı süreçlerden geçirilmesiyle üretilebilen bir enerji kaynağıdır [2-4]. Üretilen biyogaz saf bir gaz olmayıp, içeriğinde yaklaşık olarak % 55- 75 oranında metan (CH_4), % 25-45 oranında karbondioksit (CO_2), % 1-10 oranında hidrojen (H_2), % 0-0.3 oranında azot (N_2) ve % 0-3 oranında ise hidrojen sülfür gazı (H_2S) bulunmaktadır [3-4]. Küresel boyutta, enerji tüketimde kullanım oranı her ne kadar sınırlı olsa da mevcut büyük potansiyelinden dolayı her zaman alternatif bir enerji kaynağı olarak güncel kalmıştır [5-7]. Avrupa ülkelerinde özellikle de Almanya gibi tarım sektöründe gelişmiş ülkeler biyokütle atıklarından elde ettiği biyogaz enerjisini (ısı, elektrik) işletmelerinde kullanarak maliyetleri azalmaktadır.

Bu bağlamda biyogaz kullanımı; üretim aşamalarında ham madde kaynağı olarak organik atıkların kullanılması, su kaynaklarının korunmasını sağlaması, kötü koku ve hastalık oluşumunun engellenmesi gibi çevresel avantajlarda sağlamaktadır [7]. Ülkemizde yıllık ortalama 50-65 milyon ton eş değer petrol (TEP) bitkisel ve hayvansal atık potansiyeli olduğu, bahsedilen değerin ortalama % 82 oranını bitkisel atıkların oluşturduğu bilinmekte, atıkların enerji potansiyeli Türkiye'nin yıllık enerji tüketim değerinin yaklaşık % 22-27'sine denk olduğu belirtilmektedir [8-9].

Bu durum özellikle Türkiye gibi tarımsal potansiyeli yüksek olan ülkelerin, organik atıkları daha verimli bir şekilde kullanarak biyokütle enerjisine dönüştürmesini gerektirmektedir. Biyokütle enerjisinin ham maddesini bitkisel, hayvansal, evsel atıklar ve belediye atıkları oluşturmaktadır. Atıklar genellikle toprak altına gömülmekte ya da doğrudan yakılarak en verimsiz şekilde kullanılmaktadır. Organik kökenli atıkların farklı prensip ve metotlarla biyogaz enerjisi elde edilip daha yarayışlı formlara dönüştürülme

yollarına gidilmesi gereklidir. Ülkemizin önemli seviyede tarımsal atık potansiyeline sahip olması ve bu atıkların biyogaz enerjisi üretiminde kullanıldığı takdirde ülke ekonomisine büyük katkılar sağlayabileceğini gösterir [10]. Aybek ve ark. (2015)'nın yapmış oldukları çalışmada, ülkemizdeki hayvansal ve kullanılabilir bitkisel sap atıklarından elde edilebilecek biyogaz potansiyelinin yılda 331 860 PJ olduğunu ortaya koymuştur [11]. Organik kökenli atıkların enerji potansiyellerinin belirlenmesi konusunda literatürde birçok benzer çalışma yapılmıştır [12-18]. 2018 yılında yapılan bu çalışmada ise Tokat ve Bolu illerindeki mevcut kullanılabilir buğday sap atıklarının, kuru madde, uçucu kuru madde, metan gazı ve enerji potansiyelleri belirlenerek karşılaştırılmıştır.

Materyal ve Metot

Enerji potansiyelinin belirlendiği alanlar

Çalışma kapsamında Bolu ve Tokat illerinin kullanılabilir buğday sap atıklarının teorik biyokütle, biyogaz ve enerji potansiyellerinin analizi yapılmıştır.

Tokat ili

Tokat ili Orta Karadeniz kıyılarını İç ve Doğu Anadolu Bölge'lerine bağlayan önemli bir noktadadır. Kuzeyinde Samsun, kuzeydoğusunda Ordu, güneyinde Sivas, güneybatısında Yozgat, batısında ise Amasya illeri ile çevrili olan Tokat'ın toplam yüzölçümü 10 071 km²'dir. Türkiye topraklarının yaklaşık % 1.3'ünü kapsamaktadır [19].

Tokat ilinin tarım arazisi, orman, çayır-mera ve tarım dışı alan dağılımları büyüklükleri (ha) ve oluşturdukları %'lik oranları tablo 1'de verilmiştir.

Tablo 1. Tokat ili toplam arazi dağılımı

Arazinin türü	Yüz ölçümü (ha)	Toplam araziye oranı (%)*
Tarıma elverişli alan	358 139	35.56
Orman alanı	443 438	44.03
Çayır-mera alanı	120 036	11.92
Tarım dışı alan	85 587	8.50
Toplam	**1 007 200**	**100.00**

*: Hesaplanan değerler

Tablo 1'e göre, Tokat ili arazi dağılımında en büyük alan % 44.03 ile ormanlar oluştururken, en küçük alan ise % 8.50 oran ile tarım dışı alanlar teşkil etmektedir.

Bolu ili

Türkiye yüzölçümünün ortalama % 1'lik kısmını oluşturan ve yüz ölçümü 8 276 km² (827 600 ha) ile Karadeniz Bölgesi'nin Batı Karadeniz bölümünde yer almaktadır. Ortalama rakım seviyesi 1000 m olan Bolu ilinin batısında Düzce ve Sakarya, güneybatısında Bilecik ve Eskişehir, güneyinde Ankara, doğusunda Çankırı, kuzeyinde Zonguldak ve kuzey doğusunda ise Karabük İlleri yer almaktadır [20].

Bolu ilinin tarım arazisi, orman, çayır-mera ve tarım dışı alan dağılımları büyüklükleri (ha) ve oluşturdukları %'lik oranları tablo 2'de verilmiştir.

Tablo 2. Bolu ili toplam arazi dağılımı

Arazinin türü	**Yüz ölçümü (ha)**	**Toplam araziye oranı (%)***
Tarıma elverişli alan	149 664	17.68
Orman alanı	471 514	55.71
Çayır-mera alanı	124 440	14.70
Tarım dışı alan	100 820	11.91
Toplam	**846 438**	**100.00**

*: Hesaplanan değerler

Tablo 2'ye göre, Bolu ili arazi dağılımında en büyük payı % 55.71 ile ormanlar oluştururken, en küçük payı ise % 11.91 ile tarım dışı alanlar teşkil etmektedir.

Buğdayın çalışma alanlarındaki yetiştirilme miktarları (da)

2017 yılında, Tokat ve Bolu ili ve ilçeler bazında yetiştirilen buğdayın ekim alanları tablo 3'de verilmiştir.

Tablo 3. İlçeler bazında buğday ekim alanları [21]

TOKAT/İlçeler	**dekar (da)**	**BOLU/İlçeler**	**dekar (da)**
Almus	27 680.00	Dörtdivan	60 000.00
Artova	47 364.00	Gerede	100 000.00
Başçiftlik	19 470.00	Göynük	67 000.00
Erbaa	136 060.00	Kıbrıscık	4 250.00
Merkez	72 626.00	Mengen	12 900.00
Niksar	169 847.00	Merkez	130 180.00
Pazar	22 140.00	Mudurnu	79 367.00
Reşadiye	65 504.00	Seben	25 677.00
Sulusaray	43 640.00	Yeniçağa	30 000.00
Turhal	224 062.00		
Yeşilyurt	15 552.00		
Zile	393 357.00		
Toplam	**1 237311.00**	**Toplam**	**509 374.00**

Tablo 3'te ilçelere göre, verilen ekim alan değerleri kullanılarak ortalama biyokütle potansiyeli belirlenirken, buğday bitkisi için toplanabilir değer olarak % 15 oran baz alınmıştır [22]. Atıklardan elde edilebilecek kuru madde, uçucu kuru madde ve metan

gazı potansiyelleri Sharma ve ark. (1988) tarafından kullanılan yönteme göre hesaplanmıştır [23]. Uçucu kuru madde değerleri belirlenirken literatürdeki gerekli parametreler kullanılmıştır. Atıklardan elde edilebilecek metan gazının enerji değeri ise Aybek ve ark. (2015)'nın yapmış oldukları çalışmadaki yönteme göre tespit edilmiştir [11].

AP = (((EA x 37 x 15)/100)1000)

Burada;

AP, buğday atık miktarı potansiyeli (ton/yıl); *EA*, buğday ekim alanı (da).

KM = ((AP x 88)/100)

Burada;

KM, Elde edilebilir kuru madde potansiyeli (ton/yıl).

UKM = ((AP x 87)/100)

Burada;

UKM, Uçu kuru madde potansiyeli (ton/yıl).

ÖMO = UKM x 0.25

Burada;

ÖMO, Özgül metan oranı (CH_4 kg).

ME= ÖMO x 36

Burada;

ME, Elde edilebilir metan gazının enerji değeri (MJ).

Bulgular ve Tartışma

Atık ve kuru madde potansiyeli

Tokat ve Bolu illerine ait yıllık ortalama buğday atık ve kuru madde potansiyelleri hesaplanmıştır (Tablo 4).

Tablo 4. Atık ve kuru madde potansiyeli

TOKAT/İlçeler	Atık miktarı (ton/yıl)	Kuru madde (ton/yıl)	BOLU/İlçeler	Atık miktarı (ton/yıl)	Kuru madde (ton/yıl)
Almus	153.62	135.19	Dörtdivan	333.00	**289.71**
Artova	262.87	231.33	Gerede	555.00	**482.85**
Başçiftlik	108.06	95.09	Göynük	371.85	**323.51**
Erbaa	75.51	66.45	Kıbrıscık	23.59	**20.52**
Merkez	403.07	354.71	Mengen	71.60	**62.29**
Niksar	962.65	829.53	Merkez	722.50	**628.57**
Pazar	122.88	108.13	Mudurnu	440.49	**383.22**
Reşadiye	363.55	319.92	Seben	142.51	**123.98**
Sulusaray	242.20	213.14	Yeniçağa	166.50	**144.86**
Turhal	1 243.54	1 094.32			
Yeşilyurt	86.31	75.96			
Zile	2 183.13	1 921.16			
Toplam	**6 187.41**	**5 444.92**	**Toplam**	**2 827.03**	**2 459.51**

Tablo 4'e göre, Tokat'ın ilçeleri arasında buğday atıklarından elde edilebilecek en fazla kuru madde potansiyeli 1 921.16 ton ile Zile ilçesinde tespit edilmiş olup, Bolu ilinde ise en fazla 628.57 ton ile merkez ilçede tespit edilmiştir. Tokat-Bolu il ve ilçelerinde belirlenen buğday sap atıklarından elde edilebilir uçucu kuru madde ve özgül metan potansiyelleri tablo 4'te verilmiştir.

Uçucu kuru madde ve özgül metan potansiyeli

Tablo 4'te verilen atık ve kuru madde potansiyel değerleri kullanılarak ortalama uçucu kuru madde ve özgül metan değerleri hesaplanmıştır (Tablo 5).

Tablo 5. Uçucu kuru madde ve özgül metan potansiyeli

TOKAT/İlçeler	Uçucu kuru madde (ton/yıl)	Özgül metan (CH_4 kg)	BOLU/İlçeler	Uçucu kuru madde (ton/yıl)	Özgül metan (CH_4 kg)
Almus	133.65	33.41	Dörtdivan	289.71	72.43
Artova	288.70	57.17	Gerede	482.85	120.71
Başçiftlik	94.01	23.50	Göynük	323.51	80.88
Erbaa	65.70	16.42	Kıbrıscık	20.52	5.13
Merkez	350.67	87.67	Mengen	62.29	15.57
Niksar	820.11	205.03	Merkez	628.57	157.14
Pazar	106.90	26.73	Mudurnu	383.22	95.81
Reşadiye	316.29	79.07	Seben	123.98	31.00
Sulusaray	210.72	52.68	Yeniçağa	144.86	36.21
Turhal	1 081.88	270.47			
Yeşilyurt	75.09	18.77			
Zile	1 899.32	474.83			
Toplam	**5 383.04**	**1 345.76**	**Toplam**	**2 459.51**	**614.88**

Tablo 5'e göre, Tokat'ın ilçeleri arasında buğday atıklarından elde edilebilecek en fazla uçucu kuru madde ve özgül metan potansiyel değerleri sırasıyla 1 889.32 ve 474.83 ton

ile Zile ilçesinde belirlenmiştir. En az ise, Erbaa ilçesinde olup sırasıyla, 65.70 ve 16.42 ton olarak bulunmuştur. Bolu ilinde ise en fazla merkez ilçede tespit edilmiş olup sırasıyla, 628.57 ve 157.14 olarak hesaplanmıştır. Tokat-Bolu il ve ilçelerinde belirlenen metan gazının enerji potansiyelleri ise tablo 6'da verilmiştir.

Metan gazının enerji potansiyeli

Tablo 5'te verilen elde edilebilir metan gazı değerleri kullanılarak enerji potansiyelleri belirlenmiştir (Tablo 6).

Tablo 6. Enerji potansiyelleri

TOKAT/İlçeler	**Enerji potansiyeli (MJ)**	**BOLU/İlçeler**	**Enerji potansiyeli (MJ)**
Almus	1 202.88	Dörtdivan	2 607.39
Artova	2 058.27	Gerede	4 345.65
Başçiftlik	846.10	Göynük	2 911.59
Erbaa	591.27	Kıbrıscık	184.69
Merkez	3 156.07	Mengen	560.59
Niksar	7 380.06	Merkez	5 657.17
Pazar	962.13	Mudurnu	3 449.01
Reşadiye	2 846.57	Seben	1 115.83
Sulusaray	1 896.44	Yeniçağa	1 303.70
Turhal	9 736.95		
Yeşilyurt	675.84		
Zile	17 093.92		
Toplam	**48 447.39**	**Toplam**	**22 135.61**

Tablo 6'ya göre, Tokat'ın ilçeleri arasında buğday atıklarından elde edilebilecek en fazla enerji potansiyeli 17 093.92 MJ ile Zile ilçesinde olduğu en az ise, 591.27 MJ ile Erbaa ilçesinde tespit edilmiştir. Bolu ilinde ise en fazla 5 657.17 MJ ile merkez ilçede olup en az ise 184.69 MJ ile Kıbrıscık ilçesinde belirlenmiştir. Bolu ilinde belirlenen buğday sap atıklarından elde edilebilir enerji potansiyeli Tokat ilindeki enerji potansiyelinin ortalama % 45.69'u olduğu hesaplanmıştır. Elde edilen sonuçlar Tokat ilinin buğday sap atıkları enerji potansiyelinin Bolu ili enerji potansiyelinden yüksek olduğunu göstermektedir. Demir (2018)'in Kars ilinin biyokütle ve enerji potansiyelini araştırdığı çalışmasında, toplam biyokütle potansiyelinin 1 558 794 ton/yıl olduğu, belirtilen kaynaklardan yılda 76 913 077 m^3 biyogaz elde edilebileceğini ifade etmiştir [24]. Deniz ve ark. (2015)'nın yapmış oldukları çalışmalarda ülkemizdeki meyve suyu, bitkisel yağ ve et endüstrisi atık miktarlarının toplam 118 milyon ton civarında olduğu, bahsedilen atıklardan ise ortalama 25.3 milyar m^3 biyogaz elde edilebileceğini ifade etmiştir [25]. Alibaş ve ark. (2015), yaptıkları çalışmada Diyarbakır iline ait buğday, mısır, çeltik, arpa, soya, çavdar,

şekerpancarı ve darı gibi bitki atıklarından elde edilebilecek biyogaz potansiyelini 827.42 milyon m^3/yıl olarak tespit etmişlerdir [26].

Sonuç ve Öneriler

Çalışmada, Tokat ve Bolu illerine ait kullanılabilir buğday sap atıklarına göre elde edilebilecek yıllık ortalama atık, kuru madde, uçucu kuru madde, metan ve enerji potansiyelleri araştırılmıştır. Belirlenen bulgular doğrultusunda Tokat ilinde ortalama 48447.39 MJ'lük ve Bolu ilinde ise ortalama 22135.61 MJ'lük bir metan gazı enerji potansiyelinin olduğu görülmüştür. Enerji açığımızın azaltılması konusunda ülke çapında mevcut buğday sap atıklarından elde edilebilecek enerji potansiyellerinin uygulamaya aktarılmasının önemli olacağı düşünülmektedir.

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Review Article

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A Review of Approaches in Steviol Glycosides Synthesis

Nurul Hidayah Samsulrizal[1]*, Abdul Latif Noh[1], Tamil CM Sundram[1], Zarina Zainuddin[1]

ABSTRACT

Stevia rebaudiana (Bertoni) is a commercially important plant worldwide. The leaves of *Stevia rebaudiana* contain steviol glycosides which are non-caloric, high-potency sweeteners. They are suitable for substituting sucrose and other artificial sweetening agents. *Stevia rebaudiana* also has many different therapeutic uses, with antidiabetic, anti-cariogenic, antimicrobial, anticancer and antioxidative properties. Rebaudioside A and stevioside are the major glycosides produced in its leaves. However, development of new varieties of *Stevia rebaudiana* with a greater content of rebaudioside A and decreased content of stevioside is the main concern lately. This is due to rebaudioside A having a more desirable sweet flavour taste than stevioside which possesses bitter aftertaste. In respect to that many biotechnological approaches are being used for the industrial improvement and manipulation of steviol glycosides content of *Stevia rebaudiana*. Transcriptome profiling has emerged as a useful tool to identify target genes involved in the steviol glycosides biosynthesis pathway. Understanding the mechanism and biosynthesis pathway of these compounds has further helped to improve the glycosides profile by up-regulating and down-regulating the desired genes. The aim of this paper is to describe the latest development in the transcriptome profiling in *Stevia rebaudiana* as well as to discuss the methods used in this endeavour.

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Introduction

Stevia rebaudiana (Bertoni) which is also known as stevia is a shrubby and herbaceous plant species belonging to the Asteraceae family [30]. It is an indigenous plant of South America which is found to be perennial throughout the year in the highland regions of northeastern Paraguay [1, 31]. *S. rebaudiana* is the only species out of 230 others, besides *Stevia phlebophylla*, that is reported to possess the unique ability of accumulating low-calorie sweetening agents called steviol glycosides (SGs) [2]. Among the different steviol glycosides produced in stevia leaves, stevioside is the most abundant followed by rebaudioside A. These two major glycosides have the potential to become healthier replacement for table sugars as they have zero calories and a desirable taste profile, being 300 times sweeter than sucrose [3, 32]. The continuously increasing demand for SGs

[1] Department of Plant Science, Kulliyyah of Science, International Islamic University Malaysia, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia
*Corresponding Author: Nurul Hidayah Samsulrizal, e-mail: hidayahsamsulrizal@iium.edu.my

production worldwide as an alternative sugar intensifies the commercial value of this plant in both biopharmaceutical and food and beverages industries [33]. New researches have emerged by adopting various biotechnological strategies and approaches to understand, stimulate or improve the biosynthesis of these secondary metabolites in stevia. In this respect, transcriptome has gained more attention and become promising methods to assess the genetic diversity of the plant's molecular characteristics [34]. As such, transcriptome analysis is widely used to identify gene expressions that are involved in the biosynthesis of steviol glycosides in the leaf tissues and under certain circumstances as well [34]. This review aims to provide a summary on the latest development of the genetic profiles of steviol glycosides and the strategies that are involved in the transcriptomics level.

Stevia rebaudiana

Stevia rebaudiana (Bertoni) belongs to the family Asteraceae [35], or sunflower family, which is among the largest families of flowering plants, spreading across 1,620 genera and 13 subfamilies [4]. The genus *Stevia Cav.* is one of the genera within the tribe Eupatorieae which is unique due to its flower morphology [5]. It comprises of approximately 230 species of annual and perennial herbaceous, shrub and sub-shrub plants [1] that live naturally in multiple places including mountain regions, river borders and dry valleys [6]. Out of all these species, *S. rebaudiana* is the only plant that produces highly valuable sweetening agents with desirable taste profile [2]. It is a short-day plant with a critical day length of approximately 12 to 13 hours [7, 36]. However, the critical day length may vary among cultivars from as early as 8 hours to as long as 14 hours, depending on their photoperiod sensitivity [7, 8 and 9]. Stevia plant morphology includes extensive root system with brittle stems and small, elliptical leaves that occur in alternate arrangement [3, 37]. As described by Ceunen *et al.* (2012), SGs production mainly occurs in the leaves of the plant in which 33 glycosylated diterpenes of kaurene-type have been discovered whereas a lesser amount is found within its flowers and stem, and almost none in its roots [38]. Apart from the sweetening glycosides, the leaves of stevia also contain essential amino acids, minerals such as phosphorus, potassium, sodium and calcium as well as phytochemicals such as flavonoids, alkaloids, hydroxycynnamic acids and triterpenes [2, 3]. Karimi *et al.* (2017) reported that the plant's various secondary metabolites have given it a range of therapeutic properties including anti-

hypercglycaemic, anti-hypertensive, anti-oxidative, anti-microbial, anti-cariogenic and anti-carcinogenic (Table 1). It is thus referred as a sweetening plant with great pharmaceutical significance in comparison to other natural and synthetic sweeteners following its assorted chemical and nutritional constituents and their medicinal attributes [10].

Table 1 Medicinal properties in *Stevia rebaudiana* compound

Compound	Medicinal properties	Reference
Stevioside	Anti-inflammantory	[11]
	Anti-hyperglycemic	[12]
	Hypotensive	[13]
Rebaudioside (Rb) A	PTZ-induced convulsions effects	[14]
Dulcoside A	Effect on glycemic	[15]
Steviol	Renal function	[16]

Steviol Glycosides

Steviol Glycosides (SGs) are well-known secondary metabolites in stevia. They are non-nutritive, non-toxic, high-potency sweeteners with commercially important uses in biopharmaceutical, food and beverages industries [17]. The nine most common diterpenoids that have been identified in the leaf tissues of stevia include stevioside, rebaudiosides A to E, dulcosides, steviobiosides and rubusosides [18]. Among all these SGs, stevioside and rebaudioside A are classified to be the major sweeteners in stevia [39]. The measure of the sweetness quality of stevia is attributed to the ratio of stevioside and rebaudioside A content in its leaves. Stevioside is described to be most bountiful by accommodating 60-70% of the total SGs content which is two-fold the amount of rebaudioside A [40]. However, it has a slightly less sweet taste with 300 times the sweetness of sucrose [1,19]. They are also reported to be negatively correlated in which higher content of rebaudioside A that will give a more desirable taste profile since stevioside has a lingering effect of pungency and a bitter aftertaste [1]. Accordingly, these sweeteners have a great demand to be utilised as table sugar substitute since the sweetening effect of these compounds is purely taste and they are not metabolised in the human body [1]. In other words, it possesses acceptable sweetening taste at a healthy value. Therefore, it is a great alternative sugar for diabetic patients and those planning to

control their blood glycemic index since the sweet compounds can pass through the digestive system without chemically breaking down hence producing zero calories [1,41].

Biosynthesis of Steviol Glycosides

The biosynthesis of SGs partly shares a common route with gibberellins through plastidal methyl erythritol 4-phosphate (MEP) and cytosolic mevalonic acid (MVA) pathways (Fig 1). Both of these metabolites are mainly synthesised in the mesophyll cells of the leaves and almost untraceable in the roots [1, 18, 20]. The initial phase of steviol biosynthesis occurs in plastid which involves the localised MEP pathway where several enzymes consecutively work to catalyse the production of isopentenyl pyrophosphate [42]. These enzymes include deoxyxylulose phosphate synthase (DXS), deoxyxylulose phosphate reductase (DXR), 4-diphosphocytidyl-2-C-methyl-d-erythritol synthase (CMS), 4-diphosphocytidyl-2-C-methyl-d-erythritol kinase (CMK), 4-diphosphocytidyl-2-C-methyl-d-erythritol 2,4-cyclodiphosphate synthase (MCS), 1- hydroxy2-methyl-2(E)-butenyl-4-diphosphate synthase (HDS) and 1-hydroxy-2-methyl-2(E)-butenyl-4-diphosphate reductase (HDR) [18,20].

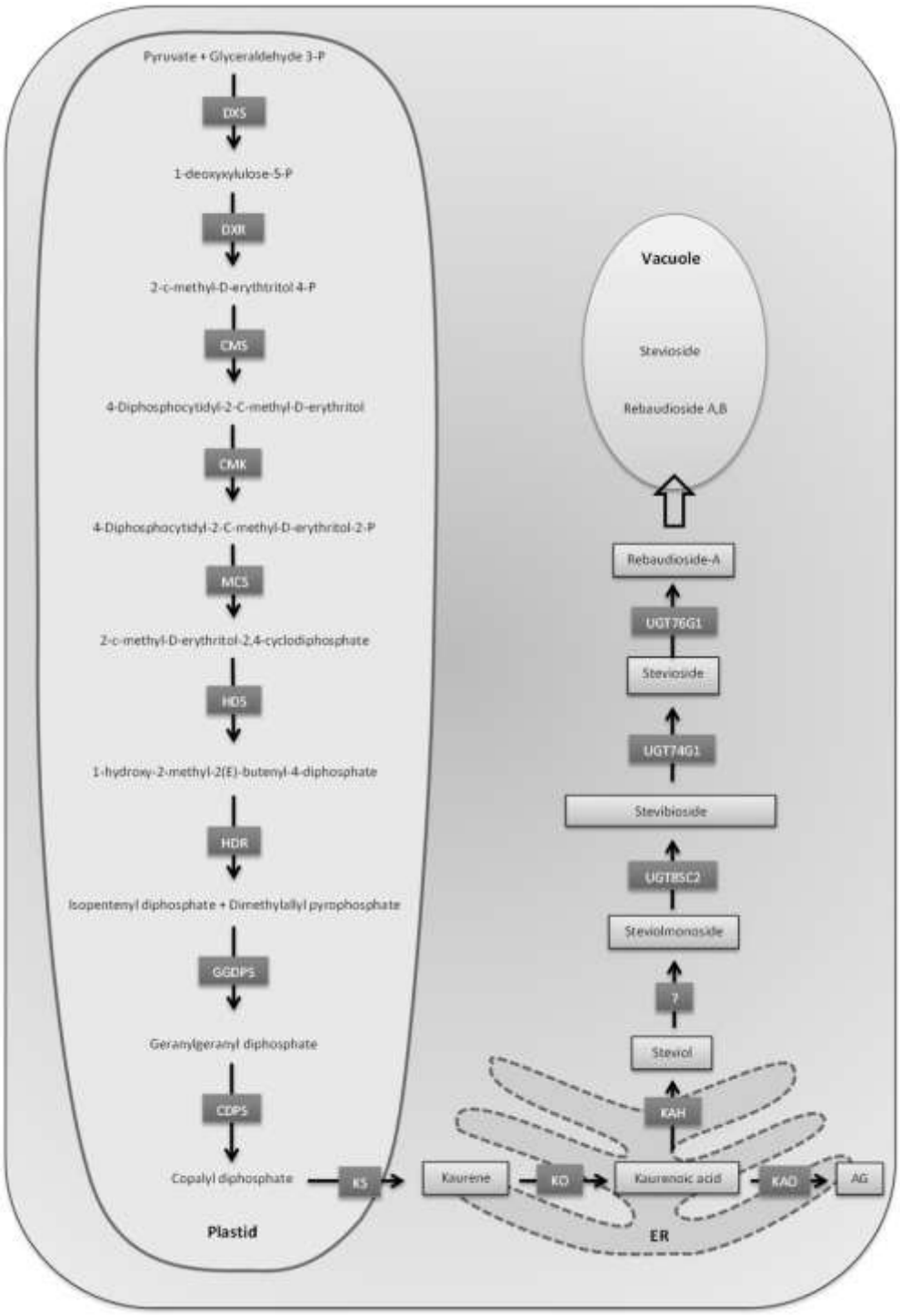

Fig 1 Diagrammatic representation of genes involved in SGs biosynthesis pathway. Abbreviations are as follows: DXS (1-deoxy-D-xylulose-5-phosphate synthase), DXR (1-deoxy-D-xylulose 5-phosphate reductoisomerase), CMS (2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase), CMK (4-diphosphocytidyl-2-C-methyl-D-erythritol kinase), MCS (2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase), HDS (4-hydroxy-3-methylbut-2-enyl diphosphate synthase), HDR (4-hydroxy-3-methylbut-2-enyl diphosphate reductase), GGDPS (geranylgeranyl diphosphate synthase), CDPS (ent-copalyldiphosphate synthase), KS (ent-copalyl diphosphate synthase), KO (ent-kaurene oxidase), KAH (ent-kaurenoic acid 13- hydroxylase), UGT85C2 (UDP-

glycosyltransferase 85C2), UGT74G1 (UDP-glycosyltransferase 74G1),UGT76G1 (UDP-glycosyltransferase 76G1), UGT? (unknown UGT), and KAO (ent-kaurenoic acid oxidase).

The second stage of the steviol biosynthesis occurs when geranylgeranyl diphosphate synthase (GGDPS) condenses four isoprene units of isopentenyl pyrophosphate to produce geranylgeranyl diphosphate which is the common precursor for the synthesis of all diterpenoids [43]. This compound is then converted into ent-kaurenoic acid by the following action of enzymes copalyl diphosphate synthase (CDPS), kaurene synthase (KS) and kaurene oxidase (KO) [20]. The steviol glycoside and gibberellin pathways diverge at kaurene where two different endoplasmic reticulum-membrane located cytochrome P450 monooxygenases (CYPs) acted to convert ent-kaurenoic acid into either steviol by kaurenoic acid hydroxylase (KAH) or gibberellic acid by kaurenoic acid oxidase (KAO) [18].

The final phase involves the glycosylation of steviol in cytosol by UDP-glycosyltransferases (UGTs) such as *UGT85C2, UGT74G1* and *UGT76G1* to form various types of steviol glycosides in which they are then vacuolated [1,18,20]. Most of the enzymes involved in the biosynthesis of SGs in stevia have been identified. Therefore, in order to increase the production of SGs, current researches should focus on metabolic engineering of these biosynthetic pathways. Silencing 3 major UGT genes through *Agrobacterium* mediated gene transformation of *S. rebaudiana* were found to increase SGs production [17].

Biotechnologıcal Approaches for Stevıol Glycosıdes Improvement in *Stevıa Rebaudıana*

Biotechnological techniques have offered wide opportunities and novel findings in the engineering of SGs biosynthesis pathway in stevia. These include all tools that can assess the transcriptomics, metabolomics and proteomics of the plant. Among these are random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), high-performance thin layer chromatography (HPTLC) and next generation sequencing (NGS) technology [21]. These methods are robust, widely applicable, fast, efficient and cost-effective [44]. They require only small amounts of template DNA to produce high information output. With evaluation of genetic diversity made possible, the genomic

resources from the generated data set have been useful to understand the mechanism and pathway of the secondary metabolites. These has been helpful to improve the sweeteners profile in stevia by up-regulating and down-regulating desired genes. For instance, new researches that focus on DNA-based molecular markers have emerged. These researches aim to establish a better understanding of the genetic variability of stevia genotypes up to the point where they can manipulate the ratio of rebaudioside A and stevioside production in the plants leaves [1].

A vast range of molecular marker technologies have also been used to develop functional molecular markers for diversity characterization and genetic improvement of the plant [18]. Apart from that molecular breeding approaches also have been implemented to increase the dry weight of stevia leaves thus increasing the sweetening compounds yields [21]. These biotechnological approaches not only have significant role in the improvement of SGs production in stevia, but can be extended in improving the plant's overall agronomy, biochemistry, evolutionary studies, genome mapping, morphology and physiology as well.

In near future metabolic engineering will be the stepping stone towards biotechnological production of SGs in heterologous host (such as *E. coli*, *S. cerevisiae*, cyanobacteria or moss). As initial step towards improving heterologous production terpenoids, *S. cerevisiae* has been successfully rewired to boost the flux via MVA pathway [22]. Meanwhile, *E. coli* has been successfully constructed to express two *S. rebaudiana ent*-kaurene genes encoding *ent*-copalyl diphosphate synthase (CPPS) and *ent*-kaurene synthase (KS) enzymes (CPPS-KS module) [45]. In this study, overexpression of three key enzymes of upstream pathway, DXS, IspA and IDI together with expression of genes encoding GGPPS from *Rhodobacter spharoides* in *E. coli* strain MG1655 co-expressing the synthetic CPPS-KS module increased the total production of *ent*-kaurene by 5 folds [23]. These four enzymes (DXS, IDI, IspA and GGPPS) have been widely reported as rate limiting enzymes of pathways of many diterpenoids [24]. Thus, overexpressing these enzymes could possibly lead to increased production of SGs.

Transcriptome Profiling of Genes Related to the Biosynthesis of Steviol Glycosides

The most significant primary step to optimise the amount of SGs produced in the leaves of stevia is by analysing the genes transcripts that are related to these metabolites' biosynthesis. According to Nature, (2018), the study of an organism's complete set of

RNA transcripts by using high-throughput modus is called transcriptomics [25]. These transcripts are fabricated either under exclusive conditions or in specialised cells by the organism's genome. Transcriptomics enable the identification of target genes that are useful for further studies in metabolic engineering besides providing a way to understand the genomics of non-model plant without reference genome like stevia. RNA-Seq, for instance, is an effective tool in NGS technology [46]. It is commonly used in researches that need to identify the genes that are expressed during SGs production through an in-depth transcript profiling. Its ability to measure transcripts in a precise manner while also being effective for annotation uses, discovery of single nucleotide polymorphisms (SNPs) and *de novo* assembly has made it a popular method in this area of research. An example of this can be seen in a study done by Chen *et al.* (2014) in which a thorough profiling of the transcriptome of three stevia genotypes (SR-1, SR-2 and SR-3) was successfully demonstrated using the combination of RNA-Seq and digital gene expression (DGE) [26]. In this study, 80,160 unigenes were annotated and 14,211 of the sequences were characterised into 250 specific metabolic pathways using Kyoto Encyclopaedia of Genes and Genomes (KEGG). The gene sequences of all the enzymes commonly related to the SGs biosynthesis were then analysed in which 143 UGTs unigenes were determined. From there, the expression patterns of eight genes, namely, *GGDPS, CPPS, KS, KO, KAH, UGT85C2, UGT74G1* and *UGT76G1*, were further evaluated using qRT-PCR which confirmed their involvement in the synthesis pathway [47].

In another study, Kim *et al.*, (2015) took a step further to elucidate the biosynthetic routes and spatial distribution of diterpenoids through the integration of metabolomics and transcriptomics [27]. This study explored the biochemical specialisation of the leaf tissues for diterpenoid production (i.e. diterpenoid glycosides and labdane-type diterpenoids) using metabolite profiling and comparative RNA-Seq transcriptomic analysis of two different tissues, trichromes and leaf without trichromes. It was performed on the basis that plant diterpenoids production and build-up only occur in specialised tissues or specific types of cell. The findings from the differential gene expressions confirmed that SGs only accumulate in leaf cells while other labdane-type diterpenoids are stored in the trichromes. Specific enzyme-encoding genes that were engaged in the initial steps of SGs biosynthesis or the MEP pathway, were identified as 1-deoxy-xylulose 5-phosphate synthases (DXS) genes, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR)

genes, 1-hydroxy-2-methyl-2-(E)-butenyl4-diphosphatereductase (HDR) genes, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (MCT) genes, 4-(cytidine 59-diphospho)-2-C-methyl-D-erythritol kinase (CMK) genes, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS) genes, and 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS) genes. This method of combining metabolomic and transcriptomic analysis has provided a comprehensive overview on the biosynthetic routes of specialised diterpenoids that are distinct to different parts of stevia leaf tissues[48].

Previous studies identified that SGs production level is high in vegetative phase until flower bud emergence, in which its level is the highest, and is followed by a diminishing amount in flowering phase [12]. Hence in a more recent study, Singh *et al.* (2017) added valuable information on the biosynthesis pathway of SGs in stevia leaves by unravelling in-depth transcriptional profiles of the genes that were involved during different developmental phase transitions (i.e. leaf tissue in vegetative phase, bud phase and flowering phase) [49]. The fact that SGs partially share a biosynthesis route with gibberellic acids and its accumulation in the leaves is ontogeny-dependent makes studying stevia at transcriptional level more complicated. Hence, this research adopted a global transcriptome sequencing approach to effectively comprehend the influence of these phase transitions towards gene expression during SGs biosynthesis. A total of 41,262 genes were annotated in which *de novo* assembled transcripts using various NGS platforms successfully detected all 46 genes that were involved in the plastidal MEP and cytosolic MVA pathways. Differential gene expression and quantitative analysis of vital genes such as *DXS*, *HMGR* and *KA13H*, and gene regulators such as *WRKY*, *MYB*, *NAC* and *TF*s showed the application of metabolic flux between SGs and gibberellic acids production during the transitions [12]. Furthermore, classification of putative candidates such as cytochrome P450 monooxygenases (CYPs) and UGTs enhanced the genomic resources. The information obtained on these candidates is useful for molecular breeding and genetic engineering efforts in order to enrich SGs content, biomass and yield.

The commercial importance of SGs has resulted in using elicitors as a potential method to induce the production of these compounds in stevia [28, 29, 51]. In a study by Lucho *et al.*, (2018), four stress-related elicitors, namely, methyl jasmonate (MeJa), spermidine (SPD), salicylic acid (SA), and paclobutrazol (PBZ) were introduced to investigate their

effects towards any changes in SGs' contents and the transcript levels of the corresponding biosynthetic genes. Six elicitor-responsive genes were discovered from SGs biosynthesis pathway i.e. *HDR*, *GGDPS*, *CDPS*, *KS*, *KO*, and *KAH*. These genes commendably can be regulated at the transcriptional level. In terms of the elicitors, MeJa and SPD were found to give positive effects in up-regulating the transcription of the genes related to SGs biosynthesis. Meanwhile, PBZ treatment was shown to down-regulate the genes that encode kaurenoid enzymes. On the other hand, SA treatment did not influence *UGT85C2*, *UGT74G1*, and *UGT76G1* transcription though it reduced the level of stevioside produced [50]. Overall, this study has offered new insights into the transcriptional response mechanisms in stevia plants under the effect of these elicitors. However, there is still a lack of information about the transcription factors and key regulators that affect the up-regulation and down-regulation of the genes involved. Therefore, studies that highly integrate transcriptomic, metabolomic, and proteomic studies should be carried out to gain better understanding of the gene regulation.

Conclusion

Among the many biotechnological approaches that are available, RNA-based study or transcriptomics has emerged as one of the promising methods to stimulate and induce SGs biosynthesis in stevia leaves. The transcriptomic profiling of genes involved in the biosynthesis route of SGs enables target genes to be identified and is useful in metabolic engineering of the plant for improvement of the compounds content, biomass and yield. RNA-Seq is a part of NGS technology that is robust, universal, cost-effective and effective to understand the genomics of non-model plant species like stevia. Information obtained through the use of this tool can enhance the understanding of the plant's genomics and assist in further development of various areas including agronomy, biochemistry, genome mapping and evolutionary studies of stevia. For future prospects, extensive researches involving "omics" technology, i.e. transcriptomic, metabolomic and proteomic studies, can be performed in stevia to understand better the underlying chemical processes especially in the regulation of genes and their conversion into functional products such as SGs metabolites and proteins.

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Research Article

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Isolation of bacteria from Tuz Gölü lake that can grow on high salt concentration

Tee Jyng Sheng[1], Yilmaz Kaya[2], Fahrul Huyop[1,3]

ABSTRACT

Today, extremophile is widely studied by the scientist due to its strong survival features that allow them to survive under extreme environment. Halophile is one example that inhabit high salt concentration environment. Isolation of bacteria from the area of Tuz Gölü lake, also known as hypersaline lake in the central plateau of Turkey, led to the isolation of 4 halotolerant bacteria, which were able to grow optimally in media with 0–10% of salt. Surprisingly, the strain A-4 isolate was successfully isolated from the Tuz Gölü lake water on the minimal media that consists of 2,2-dichloropropionic acid (2,2-DCP) as a carbon source. This indicated that the strain A-4 was very useful in the environmental remediation due to its capability to break down 2,2-DCP, a halocarboxylic found in herbicide. Further analysis such as biochemical tests and 16S rRNA sequence analysis were necessary to further identification of the species of the bacteria.

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Introduction

In the central plateau of Turkey, there is a hypersaline lake with approximately 30% of NaCl concentration. This lake known as Tuz Gölü which it is the second largest lake of Turkey with the total surface area of 1665km^2. The width of the lake is 35 km and the length of the lake is 90km [1]. Despite the large area of hypersaline lake, this lake is very shallow with the height around 0.5 to 1 metre deep. In dry summer, huge amount of water will be evaporated, leaving a 30cm thick of salt layers in the lake surface. The thick of salt layers have a high market value. Each year, the salt production from Tuz Gölü lake is estimated 300,000 tons which is equal to 60 % of the total salt production in Turkey [2]. The density of this hypersaline lake water is slightly higher than the water density. This lake water density and water density are 1.225g/cm^3 and 0.997g/cm^3 respectively. Besides that, the annual rainfall in the Tuz Gölü lake area is very low which is only 250 mm per year [3].

[1] Universiti Malaysia Pahang, Faculty of Industrial Science & Technology, Department of Industrial Biotechnology, Malaysia
[2] Ondokuz Mayıs University, Faculty of Agriculture, Department of Agricultural Biotechnology, Turkey
[3] Universiti Teknologi Malaysia, Faculty of Science, Biosciences Department, Malaysia
*Corresponding Author: Tee Jyng Sheng. e-mail: jyngsheng@gmail.com

Today's scientists have raised the interest in studying the extremophilic microorganisms such as thermoacidophiles, hyperthermophiles and hyper-halophiles [4]. The main reason of studying these organisms is to understand the biochemical mechanisms of the ability to withstand the extremophilic extreme environment. Many useful enzymes or molecules that synthesis by these organisms can be very useful in the bioremediation process, food industry and bioprocess industry. Among these extremophilic microorganisms, thermophiles have been studied and reported the most. However, halophilic bacteria have received less focus and attention in the scientific study.

In fact, the enzyme synthesis from the halophilic bacteria are very valuable due to the bacteria has high contamination resistance characteristic. Today, the cost of production and the downstream purification of enzyme from microorganisms are high because of the high energy consumption. Moreover, microbial contamination is another problem which cause a big loss to the industry. This problem will greatly affect the economy as well as the process effectiveness of the industry [5]. Hence, it is important to look for the contamination resistance microorganisms in order to reduce the energy consumption that apply for the sterilization purpose.

Recently, contamination resistant microorganisms like extremophiles which including halophilic, acidophilic, thermophilic and alkaliphilic bacteria are popular in bioproduction industry. There is successful example that applying the halophilic bacteria for the production of biopolymer, protein and chemicals. For instance, the bacteria *Halomonas campaniensis* that use in the production of poly(3-hydroxybutyrate) (PHB) was cultured and grown under an open and continuous fermentation process. Surprisingly, there is no contamination of culture for over 65 days [6]. Hence, it is crucial for the researcher to look for the novel halophilic bacteria that able to use in bioproduction industry in order to reduce the cost of operation.

Isolation of the halophilic bacteria is an important field of study because microorganisms that can grow in hypersaline environments are not only resistant to high salt concentration but also resistant to other stresses like pH, nutrients depletion and temperature [7]. For example, psychrophilic microorganisms isolated from the saline lakes in cold climate region like Arctic can grow in both extreme salt concentration and cold temperature [8]. Such surviving characteristics are very useful in the food industries, detergent and environmental bioremediation.

In order to successfully achieve this objective, the ideal place for sample collection that has been chosen is Tuz Gölü lake due to its hypersaline condition. In theory, there is difference between halotolerant bacteria and halophiles. Halotolerant bacteria can grow and survive without the need of NaCl, but they still can grow under saline condition [9]. However, halophiles cannot grow without the NaCl. In facts, there are three groups of halophiles which classified based on the responding of the halophiles to the NaCl. The first group is slight halophiles which the bacteria can grow well at 2 to 5% NaCl (0.34 to 0.85 M). The second group is the moderate halophiles with bacteria that can survive at 5 to 20% NaCl (0.85 to 3.4 M). Lastly, the extreme halophiles, a group of bacteria that can grow at 20 to 30% NaCl. (3.4 to 5.1 M) [10].

In this study, the main aim is to isolate the bacteria that can grow on high salt concentrations. Therefore, the isolated pure culture bacteria can be used to study the biochemical mechanisms or applying in the bioproduction industry in the future.

Material and Methods

Water sampling and reagent used

Hypersaline lake water samples were obtained aseptically from the hypersaline lake Tuz Gölü. In this study, distilled water was used to prepare different types of media. The chemicals used in the experiment and their brand will be listed in Table 1.

Table 1 List of chemicals used in the experiment

Chemicals	**Brand**
Dipotassium hydrogen phosphate trihydrate $K_2HPO_4 \cdot 3H_2O$	QRec
Ammonium sulfate $(NH_4)_2SO_4$	Wako
Sodium dihydrogen phosphate dihydrate $NaH_2PO_4 \cdot 2H_2O$	Wako
Iron(II) sulfate heptahydrate $FeSO_4 \cdot 7H_2O$	Wako
Manganese(II) sulfate tetrahydrate $MnSO_4 \cdot 4H_2O$	Wako
Magnesium sulfate $MgSO_4$	Sigma-Aldrich

Zinc Sulfate monohydrate $ZnSO_4 \cdot H_2O$	Wako
Nitrilotriacetic acid $C_6H_9NO_6$	Sigma-Aldrich
Cobalt(II) chloride hexahydrate $CoCl_2 \cdot 6H_2O$	Merck
2,2-dichloropropionic acid sodium salt $C_3H_3Cl_2O_2Na$	Merck

Media preparation

There were two types of media used in this experiment which were nutrient media and minimal media. A standard nutrient media was prepared which each 28 g of nutrient agar powder were suspended in a litre of distilled water.

Besides that, the preparation of minimal media involved the mixture of two different stock solutions. The first stock solution was basal salts, made of $K_2HPO_4 \cdot 3H_2O$ (42.5 g l^{-1}), $(NH_4)_2SO_4$ (25.0 g l^{-1}), and $NaH_2PO_4 \cdot 2H_2O$ (10.0 g l^{-1}). The second stock solution was made up by trace metal salts, $FeSO_4 \cdot 7H_2O$ (120.0 g l^{-1}) , $MnSO_4 \cdot 4H_2O$ (30.0 g l^{-1}), $MgSO_4$ (2.0 g l^{-1}), $ZnSO_4 \cdot H_2O$ (30 g l^{-1}), nitrilotriacetic acid (1.0 g l^{-1}) and $CoCl_2 \cdot 6H_2O$ (10 g l^{-1}) [9]. The liquid minimal media was prepared in the 250ml of conical flask. 10ml of the basal salts and 10ml of trace salt were mixed followed by the addition of 2ml of 1M 2,2-dichloropropionic acid sodium salt solution. Then, the mixture was top up to 100ml by distilled water. The preparation of a solid minimal medium was prepared by mixing liquid minimal media with 1.5% (w/v) Oxoid bacteriological agar No. 1.

All the media were autoclaved at 121°C, 15psi for 15 minutes.

Isolation of Bacteria

Isolation of bacteria were done by transferring 0.1ml of the Tuz Gölü lake water sample onto the nutrient agar and minimal media that containing 2,2-DCP followed by spread plate technique. The culture plates were incubated for 24-48 hours at 30^{o}C followed by the selection of bacterial colonies according to their distinctive morphologies. Isolated strains were further purified to obtain a pure culture using a standard microbiological technique.

NaCl tolerance test

The pure culture of different isolated strains was tested in the nutrient broth that supplemented with various concentrations of filter-sterilized solutions of NaCl (0, 35, 50, and 100 $g \cdot l^{-1}$) to check the resistant for each strain in high salinity.

Results and Discussions

There were four bacteria strains were isolated based on distinguishable features like pigment production, size and shape. These bacteria were designated as strain A-1, A-2, A-3 and A-4. Each strain was tested for morphological and NaCl tolerance. Initially, all strains were grown well on nutrient agar media incubated at 30°C over 24 to 48 hours of incubation period. The results of morphological and NaCl tolerance were summarised in Table 2 and 3 respectively.

Table 2 Morphological characterization of bacteria from hypersaline lake water in the nutrient media after 48 hours incubation

Strains	**Colony morphology**
A-1	Pale yellow, irregular, flat, entire
A-2	Yellow, circular, convex, entire
A-3	red, circular, raised, entire
A-4	Creamy white, circular, raised, entire

Table 3 NaCl tolerance test for strains A-1, A-2, A-3 and A-4 in the nutrient broth that supplemented with various concentrations of filter-sterilized solutions of NaCl after 24 hours incubation

Strains	0% (w/v) NaCl	3.5% (w/v) NaCl	5% (w/v) NaCl	10% (w/v) NaCl
A-1(Pale yellow)	+	+	+	+
A-2(yellow)	+	-	-	-
A-3(red)	+	+	-	-

A-4(creamy white)	+	+	+	+

'+'=positive grow; '-'= negative grow

Based on the morphological characterization analysis, A-3 strains had the similar morphological characteristics with the *Pseudomonas halophila HX* with red pigmentation, circular form, raised elevation and entire margin [12]. However, further test on NaCl tolerance, A-3 strain could not withstand high salinity as reported with *P. halophila HX* due to that of A-3 strain was unable to grow on 25% of NaCl (w/v) (250g l-1, 4.27M). Hence, A-3 strain was not belong to *P. halophila* group because the *P. halophila HX* can tolerate 25%(w/v) of NaCl and grow well on the media as reported earlier [12].

In this study all 4 isolates designated as strain A-1, A-2, A-3 and A4 were classified as halotolerant bacteria. They were not halophile because they were still able to grow well without NaCl in nutrient media. The presence of halotolerant bacteria in Tuz Gölü lake may likely due to the heterogenicity of the lake. The NaCl level of Tuz Gölü lake was fluctuating regularly, favouring the growth of Euryhaline microorganisms [13].

Interestingly, one isolate could grow well in the minimal media with 2,2-DCP as a carbon source. This isolate was designated as strain A-4. Since it was capable to grow on the 2,2-DCP, suggesting A-4 strain had the ability to produce dehalogenase enzyme for degradation of the organic chlorinated compound. This finding also suggested that strain A-4 could be one of the useful strains in environmental remediation. Current study elaborated the phenotypic characterization of all strains A-1 - A-4. In future further studies might involve the basic biochemical tests and the 16S rRNA sequencing.

Conclusion

Among all isolates, strain A-4 was important for further biological characterization as these bacteria were great potential source for discovery of enzymes for industrial applications or environmental remediation. Future work will be focused on identification and isolation of the potential enzymes with commercial importance.

Abbreviations

2,2-DCP: 2,2-dichloropropionic acid; NaCl: Sodium chloride; 16S rRNA: 16S ribosomal Ribonucleic acid

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Availability of data and material
Please contact the corresponding author for any data request.

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Research Article

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Analysis of WRKY Transcription Factors in Barley Cultivars Infected with *Fusarium culmorum*

Ebru Uluhan[1], Esra Nur Keleş[2], Feyza Tufan[1*]

ABSTRACT

One of the most critical problems of cereal breeding is *Fusarium* crown rot disease caused by various *Fusarium* species. *Fusarium culmorum* is one of the predominant pathogen in Turkey and causes serious product losses. In this study, the early response of barley cultivars upon *F. culmorum* infection were analyzed by disease severity and gene expression patterns of WRKY transcription factors. In that context, firstly, disease severities of 9 barley cultivars (*Hordeum vulgare* L. cvs. Epona, Escadre, Gazda, Oliver, Avcı 2002, Burakbey, Tarm 92, Manava, and Ramata) infected with *F. culmorum* were determined with disease index percentages. After 7 days of infection, Epona was more sensitive than the other cultivars while the lowest disease index was observed in Gazda. Expression analysis of *HvWRKY6*, *HvWRKY9*, *HvWRKY24*, *HvWRKY25*, *HvWRKY33*, *HvWRKY34*, *HvWRKY42*, and *HvWRKY46* genes were conducted by qPCR at 72 hours after infection in Epona and Gazda. As a result of pathogen stress, it was observed that the transcript level of *HvWRKY33* was significantly upregulated in both cultivars. *HvWRKY6*, *HvWRKY34* and *HvWRKY46* genes were increased in Epona while upregulation of *HvWRKY25* and *HvWRKY34* genes were detected in Gazda. No significant decreases were detected in any cultivars. This study is important in terms of providing an association between *WRKY* genes and pathogen stress response.

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Barley, Fusarium culmorum, Fusarium crown rot, WRKY

Introduction

When the pathogens breach the physical barriers of plants, microbial molecules and cell wall derivatives called pathogen/microbe associated molecular patterns (PAMP/MAPM) are recognized by plant membrane pattern recognition receptors (PRR) and plants initiate pattern-triggered immunity (PTI). In order to inhibit PTI, pathogens secrete virulence effectors into the cell and in turn can be recognize by intracellular plant receptors and this activates effector triggered immunity (ETI). The second level of immunity often leads hypersensitive response (HR), a localized form of programmed cell death (PCD)

[1] Haliç University, Faculty of Arts and Sciences, Department of Molecular Biology and Genetics, Beyoğlu, 34445 İstanbul / Turkey.
[2] Haliç University, Graduate Institute of Education, Program of Molecular Biology and Genetics, Beyoğlu, 34445 İstanbul / Turkey.
*Corresponding Author: Feyza Tufan, e-mail: feyzatufan@halic.edu.tr

preventing pathogen spread and systemic acquired resistance (SAR) [1]. Also, following pathogen infection, the recognition of pathogen results in the generation of reactive oxygen species (ROS). ROS accumulation is closely associated with the induction of defense response. The inducible defense response often allows to expression of a large number of defense related genes include many different types of proteins such as cell wall proteins, hydrolytic enzymes and pathogenesis related proteins, transcription factors (TF), protease inhibitors and signaling compounds (ethylene, jasmonic acid, salicylic acid etc.), enzymes associated with the synthesis of lignin and phytoalexins, and hypersensitive response [2, 3, 4]. TFs play an important role in controlling transcriptional regulation of gene expression in response to stress conditions, in cooperation with other proteins. Transcriptional reprograming is crucial for the plant defense system and help plants overcome different stresses [5]. *WRKY, bZIP, MYB, bHLH, AP2/ERF, NAC* and homeodomain TFs have been shown to participate in the regulation of stress responses [6, 7, 8, 9, 10]. WRKYs have vital roles in plant defense against abiotic and biotic stresses as well as involved in many developmental processes such as seed development, dormancy, leaf senescence, and trichome development and some signal transduction processes mediated by plant growth regulators [11, 12, 13]. WRKYs have conserved 60 amino acid regions comprising of the highly conserved *WRKYGQK* peptide sequence and a zinc finger like motif. WRKYs bind specifically to the [(C/T)TGAC(T/C)] sequence also known as W-box elements and are able to regulate expression of target genes containing these sequences in promoter regions [5]. WRKYs can act as positive or negative regulators in defense response. Numerous studies have shown that WRKYs play important role in PR gene expression and SAR-associated process [14, 15, 16, 17, 9, 18]. By the phylogenetic and comparative gene expression analysis, 45 *WRKY* family members were identified in barley [19].

Fusarium crown rot (FCR) is a destructive disease of cereals including wheat and barley. Researchers conducted in Turkey revealed that *F. culmorum* shows high prevalence among the *Fusarium* species causing the disease [20, 21]. This pathogen significantly reduces product yield and quality as well as produce mycotoxins such as deoxynivalenol and zearalenone that are harmful to human and animal health [22, 23]. In this study, firstly, we aimed to investigate the early phenotypic response of 9 barley cultivars to *F. culmorum* infection with disease index (DI) (%). Secondly, we comparatively analyzed

the expression profiles of 8 *WRKY* genes (*HvWRKY6*, *HvWRKY9*, *HvWRKY24*, *HvWRKY25*, *HvWRKY33*, *HvWRKY34*, *HvWRKY42* and *HvWRKY46*) in root tissues of Epona and Gazda cultivars at 72 hours after infection (hai).

Materials and Methods

Plant and fungal material

Seeds of barley cultivars (*Hordeum vulgare* L. cvs. Avcı 2002, Burakbey, Epona, Escadre, Gazda, Manava, Oliver, Ramata and Tarm 92) used in the study were obtained from Istanbul Yeni Yüzyıl University and commercial companies. *F. culmorum* F16 isolate obtained from the culture collection of the Department of Plant Protection, Faculty of Agriculture, Ondokuz Mayıs University, was used in pathogen stress experiments.

Pathogen infection of barley cultivars and assessment of FCR

Pathogen infection was conducted according to Covarelli et al. [24]. Briefly, seeds of barley cultivars were disinfected with 0.64 % sodium hypochlorite and 10 % ethanol for 5 min and washed three times with ddH_2O. Then, seeds were placed between filter papers soaked with ddH_2O and germinated for 2 days in dark (at 4 °C for one day then at 25 °C for 2 days). Prior to infection applications, F16 was grown for 7 days in PDA medium. The main roots of 3-day-old barley seedlings were infected with the discs of F16 and the pathogen-free discs were used as control. Seedlings were grown under controlled conditions for 7 days (25 ºC, 6/8 h of day/night cycle). Sixteen seedlings were used in each experiment with three replicates. Barley cultivars were examined phenotypically for FCR disease with browning index scale ranged from "0" to "4". DI percentages of barley cultivars were calculated using the formula DI (%) = Σ(Rn x X / 4N) x 100 (Rn: number of plants in the category; X: scale value of each plant; N: assessed for each cultivar).

Molecular analysis

Epona and Gazda cultivars were selected according to the DI% for further molecular analysis. Roots of six seedlings were sampled at 72 hai and were flash-frozen and powdered in liquid nitrogen. 50 mg of roots were used for total RNA extraction with NucleoSpin® RNA kit (Macherey-Nagel). The quantities and purities of total RNAs were determined by spectrophotometer (Multiskan GO, Thermo Scientific). First strand

cDNAs were synthesized from 500 ng of total RNA (NEB; E6300S). cDNAs were diluted 1:2 in nuclease free water for further analysis by qPCR and stored at −20 °C. qPCR was conducted using SensiFast™ SYBR No-Rox Kit (Bioline, UK) on BioRad CFX Connect™ Real-Time PCR Detection System. qPCR reactions contained 10 µl SensiFast™ SYBR No-Rox mix (1X), 0.4 µM of each primer, and 2 µL of cDNA corresponding to 25 ng total RNA. qPCR was preceded by a polymerase activation step at 95 °C for 2 min, followed by 40 cycles of 5 sec at 95 °C, 10 sec at 58 °C and 10 sec at 72 °C. Melting curve analysis was performed at the end of cycling. Two technical and two biological replicates were performed in the experiments. ADP-ribosylation factor 1-like protein and actin were examined for expression stability under pathogen stress as housekeeping genes. Primer sets of *WRKY* genes were listed in Table 1. Fold changes in gene expressions were determined by $2^{-\Delta\Delta Cq}$ method [25].

Table 1 List of primer sequences used for WRKY expression analysis by qPCR

Genes	NCBI accession	Primer sequences (5'-3')	Amplicon size (bp)
HvADP-ribosylation factor 1-like protein F	AJ508228.2	GACATCTGGTGAAGGGTTGT	95
HvADP-ribosylation factor 1-like protein R		CATTCCTCGAAGCAGTCCTC	
HvActin F	AY145451.1	GGCACACTGGTGTCATGGT	90
HvActin R		GCGCCTCATCACCAACATA	
HvWRKY6 F	EF488106.1	CGAAGGTCATTGTGCTGTTG	101
HvWRKY6 R		CTGTACCCATCGCTCATCTT	
HvWRKY9 F	DQ840408.1	AGGTTTCAGCTCATGCACCA	106
HvWRKY9 R		TGACACCCTTGCCACCACTA	
HvWRKY24 F	DQ863108.1	CATGAGCAGAGCACCATCT	110
HvWRKY24 R		GACATCATCCGCACCTGTAT	
HvWRKY25 F	DQ863109.1	CATCATGGAGGTCCAAGCAA	114
HvWRKY25 R		ACCCGACAATGTCCTTCTGG	
HvWRKY33 F	DQ863117.1	CTGCAACTTTCCCAGGTACT	96
HvWRKY33 R		GGGTCGCTGTGATCTTTCT	
HvWRKY34 F	DQ863118.1	AACCAACAGAGCGACATAGG	98
HvWRKY34 R		CTGTCGGTCTCCATCTTGAC	
HvWRKY42 F	DQ863125.1	AGTGAAGGACAGTGCTGATG	104
HvWRKY42 R		GGTCTTCCTCGTTCTCTTCC	
HvWRKY46 F	AY323206.1	ATTCGCCTGGTATGGTTGAG	106
HvWRKY46 R		TCCTCCTCCTCAGTAGCATC	

Statistical analysis

The statistical analyses related to DI % were performed using the one-way analysis of variance (ANOVA) with least significance difference (LSD) test function at $P \leq 0.05$ in R 3.1.3 statistical software with RStudio (Version 0.98.1103) and the package agricolae (**P < 0.05, ** P < 0.01, *** P < 0.001*).

Results

Pathogen infection of barley cultivars and assessment of FCR

F.culmorum hyphal growth on the root surfaces of the seedlings and necrotic formations of FCR disease were observed in all cultivars at 7 day after infection (dai). Infected seedlings were significantly suppressed in the roots and shoots compared with control groups. Among the barley cultivars infected with *F.culmorum*, Epona was selected as relatively sensitive cultivar according to the DI with 80.8 %. With a rate of 53.3 %, Gazda was a contrast cultivar with a lower DI than the other 8 cultivars (Fig. 1). A significant difference was found in Epona in terms of DI compared with other cultivars and Epona and Gazda were selected for further molecular studies.

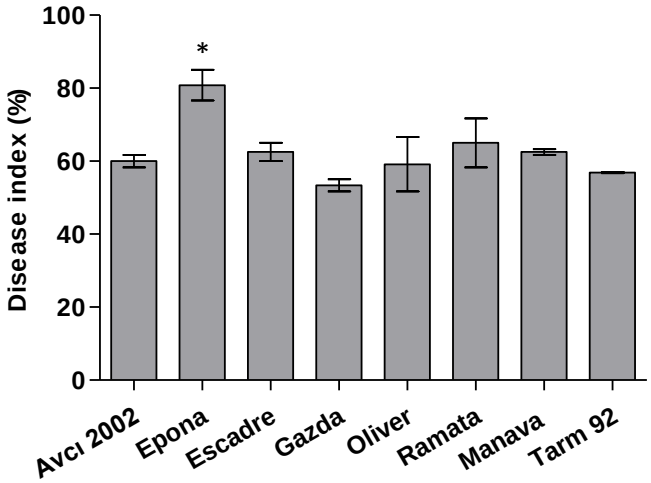

Fig 1 Determination of DI % at 7 dai in barley cultivars (**P < 0.05*)

Molecular Analysis

We have analyzed the gene expression profiles of selected *WRKY* genes at 72 hai in Epona and Gazda roots with qPCR. We compared the expression stability of two housekeeping genes (actin and ADP-ribosylation factor 1-like protein) as a reference and we found that ADP-ribosylation factor 1-like protein was more suitable in FCR for normalization of qPCR [26]. According to qPCR results, significant increases were detected in expression of *HvWRKY6* (4.6 fold), *HvWRKY33* (7.9 fold), *HvWRKY34* (2.7 fold) and *HvWRKY46* (2.6 fold) in Epona upon infection while there was a small but not significant decreases in expressions of *HvWRKY9*, *HvWRKY24* and *HvWRKY42*. In terms of Gazda, the transcript levels of *HvWRKY9, HvWRKY42, and HvWRKY25* showed a slight, but not a significant decrease in stress groups compared to control. However, *HvWRKY25* (7.7 fold) and *HvWRKY33* (5.7 fold) transcript levels were significantly increased in Gazda (Fig 2). Transcript level of *HvWRKY33* was significantly upregulated in both cultivars.

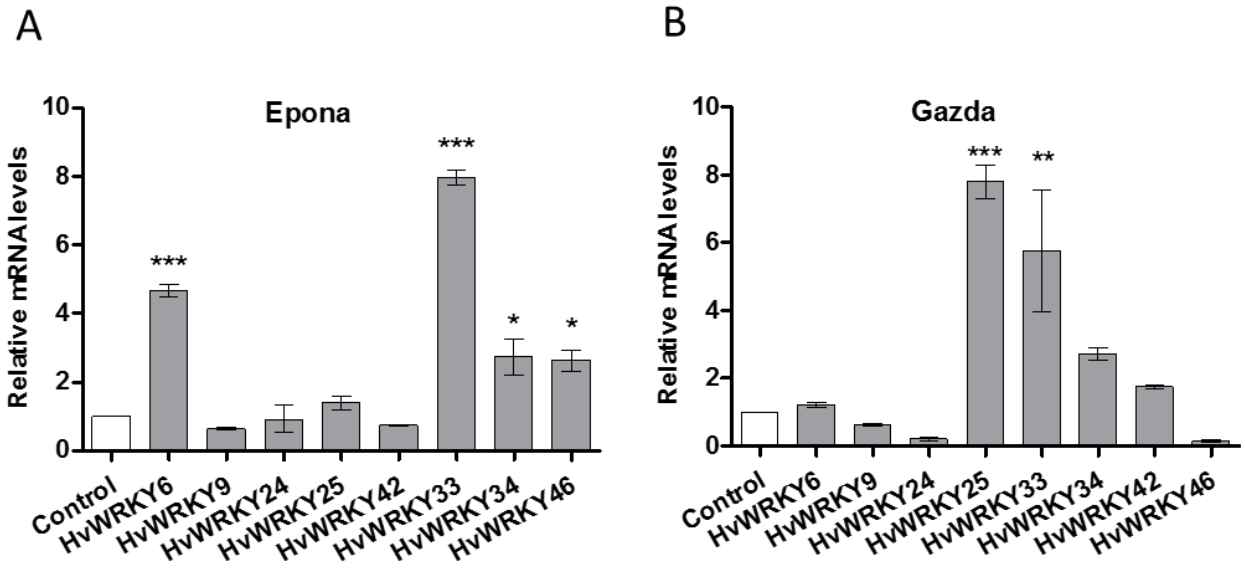

Fig 2 Determination of relative mRNA levels of *HvWRKY* in root tissues of Epona (A) and Gazda (B) at 72 hai (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

Discussion

F. culmorum is a destructive pathogen which causes FCR especially in small grain cereals such as barley and wheat. FCR reduces product yield and quality, as well as contamination of grains with mycotoxins pose serious threats to human and animal health. The use of fungicides in the chemical prevention of FCR is not an effective approach

because of the fact that pathogenic fungi have large populations and are highly immune to these fungicides. At the same time, these fungicides can cause problems such as environmental pollution and phytotoxicity [27]. *Fusarium* tolerant cultivars are the most effective and economical approach. However, there are limited reports on germplasm screening [28, 29]. Development of tolerant cultivars requires time and resistance is not permanent as pathogen often evolves to overcome host resistance. WRKYs can be successfully used in marker assisted selections to develop new cultivars with improved FCR tolerance. In this study, we firstly analysed the early responses of barley cultivars to *F. culmorum* with the infection of roots via plate assay. The assay is successfully used in several studies including wheat, barley and oat [30, 31, 32, 33].

WRKYs play important roles by controlling the expression of genes involved in various biological processes and biotic and abiotic stress responses [34, 9, 35]. There are limited reports on WRKYs on biotic stress response in barley. In a previous study by Meng et al. time-course expression profiles of 26 *HvWRKY*s including *HvWRKY6*, *HvWRKY9*, *HvWRKY42* and *HvWRKY46* were analyzed to investigate their role in mildew locus a (Mla)-mediated immunity to *Blumeria graminis* f. sp. Hordei. They found that 12 *HvWRKY*s were differentially expressed: with 10 highly upregulated and 2 significantly downregulated [36]. They conducted loss- and gain-of-function studies and demonstrated that *HvWRKY10*, *HvWRKY19* and *HvWRKY28* positively regulate the barley transcriptome in response to *B. graminis* infection. Liu et al. demonstrated that *HvWRKY1* and *HvWRKY2* repress the activity of the powdery mildew-induced promoter of *HvGER4c* [37]. In another study on *Fusarium* head blight (FHB) in barley, *HvWRKY23* was shown to modulate defense response and enhance resistance against FHB [38]. We analyzed *F. culmorum* related biotic stresses on the relative mRNA levels of 8 WRKYs in two barley cultivars Epona and Gazda. Regarding to our qPCR results, *HvWRKY33* was significantly upregulated in both cultivars. There are no previous reports regarding the effect of the *HvWRKY33* on stress response. In a previous study by Gao et al. *HvWRKY6*, *HvWRKY40* and *HvWRKY70* have exerted positive effects on wheat resistance to *Puccinia triticina* [39]. In our study, *HvWRKY6* was significantly increased upon infection in a relatively sensitive cultivar Epona while no significant change was detected in Gazda. *OsWRKY82* is orthologous with *HvWRKY6* and in a previous report was shown to induced by inoculation with *M. grisea* and *Rhizoctonia solani* [40].

Conclusion

Pathogen tolerance responses involved in complex transcriptional networks and the underlying mechanisms are largely unclear. These results may be helpful for defense response regulation by *WRKY*s upon pathogen infection in barley and determination of FCR tolerant cultivars. Further gene silencing and over-expression studies will contribute our understandings on the role of *WRKY*s in defense response to pathogen tolerance.

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Research Article

Khatri, N., et al., Effect of Different Wheat Variety and Sowing Methods on Grain Yield of Wheat under Bhairahawa Condition of Nepal. International Journal of Life Sciences and Biotechnology, 2019. 2(3): p. 175-182.

Effect of Different Wheat Variety and Sowing Methods on Grain Yield of Wheat under Bhairahawa Condition of Nepal

Narayan Khatri[1]*, Bisheshwor Prasad Pandey[1], Mamata Bista[2], Dev Lakshmi Ghimire[1]

ABSTRACT

A field experiment was conducted during winter seasons of 2016/17 and 2017/18 at National Wheat Research Program, Bhairahawa, Rupandehi, Nepal with the objective to study the different sowing methods in wheat. Variety comprises of Vijay and Bandganga were allocated in main plot whereas three sowing methods System of wheat intensification (SWI) at 20 cm × 20 cm, Line sowing at 25 cm × continuous and broadcasting were allocated in sub-plot under split plot design with four replications. Bandganga variety gave higher grain yield as compared to Vijay variety. SWI at 20 cm × 20 cm recorded significantly higher tillers (267 and 251 $m^{-2)}$ as compared to line sowing at 25 cm and broadcasting during both the years. SWI at 20 cm × 20 cm spacing recorded significantly higher grain yield (3739 and 3840 kg/ha) during 2016/17 and 2017/18, respectively. But it was found at par with line sowing at 25 cm x continuous method. Economic analysis of different sowing methods showed that both line sowing and SWI were found superior over broadcasting method. Highest Net returns of NPR 72,294 and 62,644 and B: C ratio (1.75 and 1.43) was obtained from line sowing and SWI methods respectively. Thus, SWI at 20 cm × 20 cm and line sowing method could be a better production technology to enhance the wheat productivity if mechanization is done to replace the required labor.

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Introduction

Wheat (*Triticum aestivum* L.) is grown across a wide range of environments around the world and has the highest adaptation among all the crop species. Wheat is rich source of protein, minerals and vitamins amongst all the cereals. It contributes about 60 percent of daily protein requirement and more calories to World human diet than any other food crops. It is the second most important crop after rice in Terai, Nepal, serving as one of the major staples in the diet eaten in the form of bread. There has been stagnation in wheat productivity since 2002 in Nepal. The current national average wheat yield is 2.50 t/ha [1]. The causes of low productivity is governed by many biotic and abiotic factors like,

[1] National Wheat Research Program, Bhairahawa, Rupandehi, Nepal
[2] Regional Agriculture Research Station, Lumle, Kaski, Nepal
Corresponding Author: Narayan Khatri, e-mail: narayan.iaas068@gmail.com

weather (low and high temperature stress, high RH and drought), lack of suitable high yielding genotypes for specific production domain; poor performance of existing cropping system and technologies of production being followed. Number of genetic and external factors controls the ultimate yield of wheat crop. An optimum level of single factor will not cause any appreciable increase in the yield itself, but a combination of factors contributes to the ultimate yield of wheat. It is well recognized that by keeping proper row spacing and input like seed treatment, fertilizer and seed rate etc. have an effective role in increasing the yield of crops. Wheat is generally planted by broadcast method by most of the farmers in Nepal, only progressive farmers and research scientists use line sowing. Now a day due to infestation of weeds, labor scarcity and partial mechanization in Nepal, line sowing is being practiced with proper row spacing, which besides facilitating inter-culture and convenient herbicide application for effective and effective weed control; help in intercropping and reducing the seed rate per hector without any adverse effect on the final grain yield.

The increasing demand for food necessitates further intensification of the crop productivity systems. The System of wheat intensification (SWI), based on low-tech methods, may be more labor-intensive than traditional techniques, but it requires less seeds, water, pesticide and fertilizer. Yield obtained in SWI is double than that of obtained conventional methods [2]. SWI techniques by utilizing minimum inputs and low seed rate coupled with efficient water saving (30 %) could address the problem of low productivity [3]. Therefore, this study was carried out to increase farmers' income by increasing production per unit area through the development of most profitable and productive technology in western region of Nepal.

Material and Methods

An experiment was conducted during winter seasons of 20167/17 and 2017/18 at the farm of National Wheat Research Program, Bhairahawa, Nepal. The climate of experimental site was sub-tropical with elevation of 105 masl, where maximum temperature goes up to 44.6 ^{O}C. The experiment was laid out in split plot design including variety viz; Vijay and Banganga as main plot and different sowing methods viz; broadcasting, line sowing (25 cm × continuous line sowing) and system of wheat intensification (SWI) at 20 cm × 20 cm as sub plot factor with four replication. Under SWI, two seeds per hill were sown at 20 cm × 20 cm spacing while in broadcasting at

150 kg/ha (farmers practice) and line sowing at 120 kg/ha seed rate was applied. Fertilizer was applied at 150:50:50 N_2:P_2O_5:K_2O for line sowing and 100: 25: 20 N_2:P_2O_5:K_2O kg/ha for broadcasting. Other intercultural operations were done as per recommended practices. Under SWI, 400 ml cow urine, 225 g vermicompost and 110 g of jaggery were added per liter of water and thoroughly mixed with seed containing water. The mixture was left for 6-8 hours and then filtered. The filtered seeds were treated with bavistin 2.5 g kg^{-1} seed and kept in wet jute bag for 8-10 hours. The seeds were dried in shade for half an hour just before sowing to facilitate easy sowing of seeds [4].

The crop was sown on November 23, 2016 and November 19, 2017 with a plot size of 3 m x 4 m. Full dose of phosphorus, Muriate of potash and half dose of Nitrogen were applied to wheat as basal dose at the time of sowing. The remaining dose of N was top dressed at 30 Days after sowing. To workout the economics of different sowing method treatments information on the existing market price of seed, fertilizer and herbicides were used. Cost of labor was calculated by taking into account the prevailing labor wages at the time of investigation. Gross returns, net returns and benefit cost ratio were worked out by using the following formulae and expressed in Nepalese rupee (NPR).

Gross return= Grain yield x market rate of grain

Net returns= Gross returns- total cost of cultivation

$$\text{Benefit Cost ratio} = \frac{\text{Gross returns}}{\text{Total cost of cultivation}}$$

The weather data during wheat growing season was presented graphically in Fig 1and 2. All recorded data were analyzed through GENSTAT statistical package and treatment means were compared using least significant difference (LSD) test at $P \leq 0.05$.

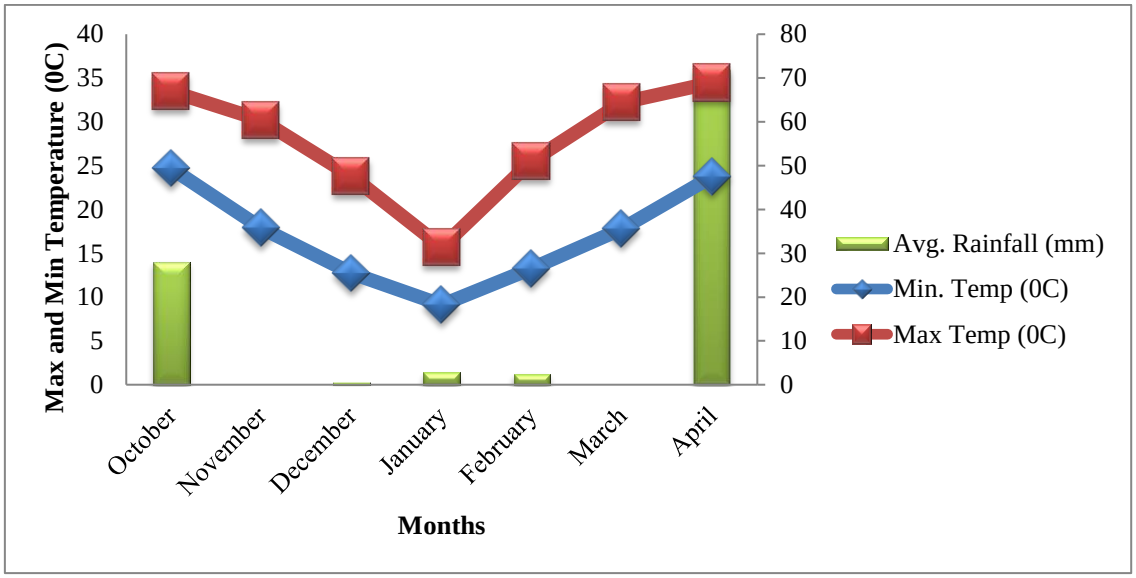

Fig 1 Weather data during wheat growing season, 2016/17

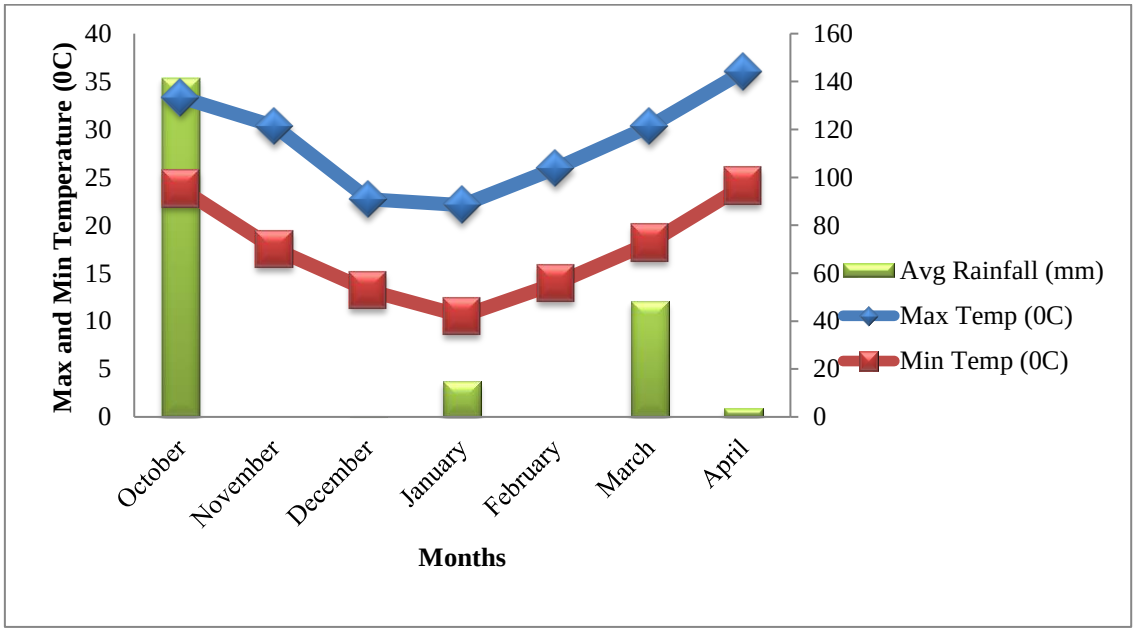

Fig 2 Weather data during wheat growing season, 2017/18

Results and Discussion

Effect of varieties

There was significant effect of variety on number of tillers per square meter but non significant effect was observed in plant height, panicle length, thousand grain weight and grain yield in both years (Table 1). Significantly highest numbers of tillers per square meter was recorded by Bandganga variety. However higher grain yield was recorded by Bandganga in comparison to yield of Vijay. Highest grain yield of

Bandganga might be due to higher number of tillers per square meter, panicle length and thousand grain weights. Combined analysis of both years' data also revealed the same results (Table 2). So, both tested improved wheat varieties; Vijay and Bandganga can be grown successfully under different sowing methods.

Effect of sowing methods

Plant height, number of tillers per square meter, thousand grain weight and grain yield were significantly different among different sowing methods while panicle length was not found significantly different (Table 1). SWI wheat sown at 20 cm × 20 cm spacing recorded significant higher tillers (267 and 251 m^{-2}) during both years but it was at par with line sowing (Table 1).

Table 1 Plant growth, yield and yield attributes of wheat as influenced by variety and sowing methods during 2016/17 and 2017/18

Treatments	Plant Height (cm)		Panicle Length (cm)		Number of tillers/m^2		1000 grain weight (gm)		Grain yield (kg/ha)	
	2016/17	2017/18	2016/17	2017/18	2016/17	2017/18	2016/17	2017/18	2016/17	2017/18
Variety										
Vijay	81	80	11	11	195	222	51.7	41.7	3539	3070
Bandganga	83	83	12	12	285	226	51.5	42.8	3762	3291
F-test	ns	ns	ns	ns	*	ns	ns	ns	ns	ns
LSD (0.05)					63					
Sowing Methods										
Broadcasting	77	77	11	11	217	174	50.6	40.3	3533	2293
Line sowing (25 cm × continuous)	78	79	11	12	235	247	50.8	43.1	3680	3408
SWI (20 cm × 20 cm)	91	90	12	12	267	251	53.5	43.3	3739	3840
F-test	**	**	ns	ns	*	**	ns	*	ns	**
LSD (0.05)	5.8	5.8			38	45.23		1.9		506.8
Interaction										
F-test	ns	ns	ns	ns	ns	ns	ns	ns	ns	
Grand Mean	82	82	11	12	240	224	51.6	42.2	3651	3180
CV %	5.3	5.3	8.2	8.1	12	15.2	7.6	3.3	11.3	12

*** and *denotes significant at 1 % and 5% ns denotes non-significant*

Higher number of tillers m^{-2}, panicle m^{-2} and tillers $hill^{-1}$ obtained in rice planted under SRI method as compared to farmers practices [5]. Significantly higher thousand grain weight was recorded in SWI methods irrespective of spacing as compared to line sowing and broadcasting (Table 1). This may be due to the wider spacing and proper aeration under SWI method. The results revealed that there was significant difference in grain yield during 2017/18 and combined analysis of both year data also reflected the same results. SWI method of wheat sowing at 20 cm × 20 cm spacing recorded significantly higher grain yield (3840 kg/ha and 3789 kg/ha) during 2017/18 and combined analysis of 2016/17 and 2017/18 over other treatments (Table 1 and 2) but it was at par with line sowing . Similar results has also been reported by [6], from his experiment at farmers' fields at Sindhuli, Nepal where wheat variety 'Bhrikuti' has yielded 2.6, 2.4 and 2.3 kg/plot of 4 m^2 size i.e. 6.5, 6.0 and 5.75 t/ha in SWI, line sowing and broadcasting methods respectively.

Table 2 Plant growth, yield and yield attributes of wheat as influenced by variety and sowing methods (Combined analysis of 2016/17 and 2017/18)

Treatments	Plant Height (cm)	Panicle Length (cm)	Number of tillers/m^2	1000 grain weight (gm)	Grain yield (kg/ha)
Variety					
Vijay	81	11	208	46.7	3304
Bandganga	83	12	255	47.1	3527
F-test	ns	ns	*	ns	ns
LSD (0.05)			30		
Sowing Methods					
Broadcasting	77	11	195	45.9	2913
Line sowing (25 cm × continuous)	79	11	241	46.8	3544
SWI (20 cm × 20 cm)	91	12	259	48.0	3789
F-test	**	ns	**	ns	**
LSD (0.05)	5.3	0.4	35.68		300.8
Interaction					
F-test	ns	ns	ns	ns	ns
Grand Mean	82	11	232	46.9	3416
CV %	4.9	7.2	9.9	5.8	6.6

*** and *denotes significant at 1 % and 5% ns denotes non-significant*

Grain yield of wheat to the tune of 4.4, 3.5 and 2.0 t/ha with SWI at 25 cm × 25 cm plant spacing, line sowing at 25 cm and wheat sown under broadcast method respectively [7]. The Aga Khan Rural Support Program, working in farmers' fields at

Bihar has reported grain yield of wheat to the tune of 3.48 t/ha in SWI as compare to usual practice (2.63 t/ha) [2]. Perusal of results of present research in light of reports available from various agencies it may be inferred that the SWI methods are slightly superior than conventional line sowing and broadcasting methods of wheat with improved recommended practices and far superior to usual farmers practice.

Economics analysis

Combined analysis showed that line sowing of wheat at 25 cm was found to be more economical than SWI and broadcasting methods. Line sowing at 25 cm fetched net returns of NPR. 72,294 with a net benefit cost ratio of 1.75 (Table 3). It may be due to the fact that requirement of manual labor for sowing of wheat under SWI is much higher and labor shortage at the time of sowing is becoming a major constraint. Sowing of wheat by SWI method emerges to produce more grain yield but considering the cost and benefit of production it is not economical than line sowing until mechanization is done in SWI to replace required manual power.

Table 3 Economic returns and cost of cultivation of different Sowing methods of wheat cultivation (Combined analysis of 2016/17 and 2017/18)

Sowing Methods	Total Cost (NPR/ha)	Gross Returns (NPR/ha)	Net Returns (NPR/ha)	B:C Ratio
Broadcasting	42,680	87,390	44,710	1.05
Line sowing (25 cm × continuous)	41,376	1,06,320	72,294	1.75
SWI (20 cm × 20 cm)	43,676	1,13,670	62,644	1.43

Conclusion

The conclusion drawn from the study shows that wheat sown under system of wheat intensification (SWI) at 20 cm × 20 cm spacing is better than line sowing at 25 cm and broadcasting methods in terms of grain yield. However, it is not economical than line sowing due to higher cost incurred in labor. Therefore, mechanization in SWI should be developed in order to replace required manual power and get higher profit.

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Availability of data and material
Please contact the corresponding author for any data request.

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Derleme / Review

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Fitoöstrojenik Bitkiler; Ne Kadar Tüketilmeli?

Rabia Vildan Soldamli[1], Sahane Funda Arslanoglu[1]

ÖZET

Yapılan çalışmalarda; soya tüketiminin menopoz sorunlarını ve kanser riskini, sarımsak tüketiminin postmenopozal dönemde kolon kanserini %50 oranında azalttığı, jinekolojik hastalıkların tedavisinde kullanılabileceği belirtilmiştir. Ayrıca; uzun süre fitoöstrojen bakımından zengin yemlerle beslenen koyunlarda güç doğum oranlarında artış, koyun ve kuzularda yüksek ölüm ve kalıcı kısırlık gibi önemli sorunlar saptanmıştır. İnsan ve hayvanlar tarafından tüketilen besinlerin bazıları östrojen hormonu salgısını artıran bileşikler içermektedir. Bitkilerde doğal olarak bulunan bu bileşikler fitoöstrojen olarak tanımlanmaktadır. Fitoöstrojen tam anlamıyla östrojen olmamakla birlikte bir yere kadar östrojenin yerine geçebilmektedir. Fitoöstrojenler doğada farklı yoğunluklarda pek çok bitkide bulunmaktadır. Fitoöstrojenler; flavonoid olanlar ve olmayanlar olarak iki gruba ayrılır. Flavonoid grubunda olan fitoöstrojenler, isoflavonlar, kumestanlar ve prenil flavonoitler olup bu grubun en önemli besin kaynakları; soya ve soya ürünleri, çay, kırmızı şarap, baklagiller, brüksel lahanası ve ıspanak gibi bitkilerdir. Flavonoid olmayan grupta ise lignanlar yer alır. Bitkiler arasında tohumu doğrudan tüketilen keten iyi bir lignan kaynağı olup önemli bir fitoöstrojenik özelliğe sahiptir. Fitoöstrojenlerin; antikanser, menopoz semptomlarını azaltma, osteoporoz, kardiyovasküler hastalıkların önlenmesinde ve antikarsinojenik olmak üzere pek çok etkisi vardır. Östrojen hormonu hem kadınlarda hem de erkekte bulunan ancak erkeklerde çok düşük düzeylerde olan bir seks hormonudur. Temel yapı taşı kolestrol olan östrojen yumurtalıklardan ve böbreküstü bezlerinden salgılanır. Kadınlarda üreme fonksiyonları, menstrüel döngü ve menopoz periyodu üzerinde önemli etkileri vardır. Dolayısı ile tüketilen gıdalar insan metabolizması tarafından salgılanan hormonlar üzerine doğrudan etkilidir. Bu makalede beslenmede kullanılan fitoöstrojenik bitkilerin tüketiminde dikkat edilmesi gereken hususlar, tüketilen fitoöstrojenik bitkiler ve etkileri incelenmiştir.

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ANAHTAR KELİMELER
Bitkiler,
İsoflovanlar,
Lignan,
Fitoöstrojen,
Menopoz

[1] Ondokuz Mayıs Üniversitesi Ziraat Fakültesi Tarla Bitkileri Bölümü, 55139 Atakum-Samsun, Turkiye.
e-mail: farslanoglu@omu.edu.tr

Phytoestrogenic Plants; How Much Should Be Consumed?

ABSTRACT

In studies shown that soybean consumption reduces menopausal symptoms and cancer risk, the garlic consumption decreases colon cancer by 50% in postmenopausal period, and it can be used in the treatment of gynecological diseases. At that time, in sheep fed with foods rich in phytoestrogen for a long time, significant problems such as increased birth rates, high death in sheep and lambs and permanent infertility were determined. Some of the foods consumed by humans and animals include compounds that increase estrogen hormone secretion. These naturally occurring compounds in plants are phytoestrogens. Phytoestrogens are found in many plants in nature. Phytoestrogen can replace estrogen, although it is not estrogen at all. The source of phytoestrogens are divided into two groups: flavonoids and non-flavonoids. The phytoestrogens in the flavonoid group are isoflavones, coumestans and prenyl flavonoids, which are the most important food sources of this group; soy and soy products, tea, red wine, legumes, brussels sprouts and spinach. The non-flavonoid group contains lignans. Directly consumed seed within plants, flax is a good source of lignans and has an important phytoestrogenic property. Phytoestrogens; there are many effects including anticancer, reduction of menopausal symptoms, osteoporosis, prevention of cardiovascular diseases and anticarcinogenicity. The estrogen is a sex hormone found in both men and women. However, this hormone is very low in men. The main building block of estrogen is cholestrol. It is secreted from the ovaries and the adrenal glands. Estrogen has important effects on reproductive functions in women, menstrual cycle and menopause period. Therefore, consumed foods are directly effective on hormones secreted by human metabolism. In this article, phytoestrogenic plants used in nutrition should be considered, consumed phytoestrogenic plants and their effects are examined.

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Giriş

Hormonlar; üreme, büyüme, gelişme, enerji üretimi ve depolanmasını düzenleyen moleküllerdir [1]. Kimyasal özelliklerine göre; glikoproteinler, polipeptidler, steroidler ve aminler olarak 4 gruba ayrılırlar [2]. Glikoproteinler, polipeptid iskeletlerine kovalent olarak bağlı oligosakkarit (glikan) zincirlerini içeren proteinlerdir [3, 4]. Glikoproteinlerde yer alan monosakkaridler; glikoz, galaktoz, mannoz, fruktoz, N-asetil glikozamin, N-asetil galaktozamin, N-asetilnöraminik asit (sialik asit) olup daha az miktarda arabinoz ve ksiloz 'a da rastlanabilir [5]. Kolestrolün sadece küçük bir kısmının değişikliğe uğramasıyla oluşan steroid hormonlar üreme fonksiyonlarını düzenlemenin yanı sıra; sinir, iskelet ve kardiyovasküler sistemler üzerine de etkilidir [6]. Glikokortikoidler, mineralokortikoidler, androjenler, östrojenler ve progesteron olarak gruplandırılan steroid hormonlar; adrenal korteks, over, testis, plesanta ve beyinde normal üreme fonksiyonu ile vücut homeostazisinde kullanılmak amacıyla üretilirler [7]. Bu hormonlar arasında en sık adı geçen "Östrojen" hormonu insanlarda yumurtalıktan, adrenal korteksten, testislerden ve plasentadan [8], düşük düzeylerde; karaciğer, adrenal

bezler ve memede de sentezlenir [9]. Bu hormonlar hipotalamus tarafından GnRH üzerinden, hipofiz ön lobunda ise FSH ve LH tarafından kontrol edilmektedir [10, 11]. Hormonal steroid bileşiklerden bir grup olan östrojen hormonu hem kadın hem de erkeklerde bulunmakla birlikte üreme yaşındaki kadınlarda oldukça yüksek seviyelerdedir [9] ve meme büyümesi, kıllanma gibi dişi sekonder karakterlerin oluşumunda rol oynar [8]. Östrojen kaynakları özellikle menopoz sonrası dönemdeki kadınlar için önemlidir Doğal olarak oluşan östrojenler; kolestrolden sentezlenen 18-karbonlu steroidlerdir. Bunlar; 17β-östrodiol (E_2), östron (E_1) ve östriol (E_3) olarak üç şekilde gruplandırılır. Bu östrojen grupları arasında kadınlarda en yaygın olarak bulunanı östrodioldür. Östron; östrodiolden daha zayıf etkiye sahip olup post-menopozal dönemdeki kadınlarda östrodiol ile karşılaştırıldığında daha yüksek düzeylerde bulunur [9]. Yapılan bir çalışma β-östrodiolün; östrondan 12, östriolden ise 80 kat fazla östrojenik etkiye sahip olduğunu göstermiştir [12]. Östrojenler; albümin, özgül östrojen ve progesteron bağlayıcı globülinlere zayıf şekilde bağlanarak kanda taşınırlar ve çok hızlı bir şekilde (30 dakika) dokulara geçerler. Karaciğer; östrojenlerin bir kısmını glukoronoidler ve sülfatlara bağlayarak safraya bırakır. Geri kalanı ise idrarla dışarı atılır. Etkili östrojenler olan östradiol ve östron karaciğer tarafından etkisi daha az olan östriole çevrilir [13, 14, 15, 16].

Fitoöstrojen

Fitoöstrojen kelimesi, Yunanca bitki anlamına gelen "*phyto*" ile dişi üreme hormonu anlamındaki östrojen kelimelerinin birleşmesinden oluşur [17]. Fitoöstrojenler, fonksiyonel olarak memelilerdeki östrojenik aktiviteyi harekete geçiren ve yapısal olarak memeli östrojeni 17β-östradiol (E_2)'e benzer özellikte [18, 19] olup; kanser, kalp hastalıkları, menopozal semptomlar ve osteoporozun önlenmesinde önemli işlevlere sahiptir [20, 21, 22]. Bu nedenle; soya ağırlıklı beslenen Asya topluluklarında düşük kardiyovasküler hastalıklar, güçlü kemik yapısı, göğüs kanseri oranı ve ateş basmalarının az olmasının nedeni fitoöstrojenlere bağlanmaktadır [8, 23, 24, 25].

Fitoöstrojenler, bitkilerde genistein, daidzein ve glisitin gibi şeker moleküllerine bağlıdır ve bu durumda biyolojik aktivite göstermezler. Vücuda alındıktan sonra bağırsaklarda bakteriyal β-glikozidazlar tarafından hidroliz edilirler ve şeker grupları ayrılır. Böylece bağırsak bakterileri tarafından biyoaktif formları olan daidzein, genistein gibi aglukonlarına dönüştürürler ve bağırsaklardan hızla emilirler. Aglukonlar bağırsakta

emildikten sonra karaciğerde glukorunit ile konjuge olurlar. Konjugantlar safra ile atılabilir, enterohepatik siklüsle reabsorbe edilebilir ya da değişmeden idrarla atılabilir [21, 26, 27]. Fitoöstrojenler hızlı bir şekilde yıkılarak vücuttan kısa sürede uzaklaştırıldıkları için uzun yarılanma ömrüne sahip ve vücutta birikebilen, endokrin sistem toksisitesine neden olabilen çevresel östrojenik kimyasallardan farklılık göstermektedirler [28].

Doğada Fitoöstrojen Kaynakları

Fitoöstrojen bakımından zengin olan bitkiler çoğunlukla *Fagales, Cucurbitales, Fabales* ve *Malpighiales* familyalarında yer almasına rağmen bazı farklı familyalardaki bitkilerde de bulunur. Helvacı kabağı, kenevir, soya, meyan kökü, kırmızı üçgül ve keten tohumu, akşam sefası, dong quai, ginseng, hayıt östrojenik aktivite gösteren bitkiler olarak bilinir [29].

Fitoöstrojenler, Tablo 1'de görüldüğü gibi flavonoid olanlar ve flavonoid olmayanlar şeklinde iki gruba ayrılır [30].

Tablo 1 Fitoöstrojenlerin Sınıflandırılması [30]

Flavonoidler			**Flavonoid Olmayanlar**
Izoflavonlar	**Kumestanlar**	**Prenil Flavonoidler**	**Lignanlar**
Genistein	Kumestrol	8-Prenilnaringenin	Larisirezinol
Daidzein		6-Prenilnaringenin	İzolarisirezinol
Glisitin		Ksanthumol	Matairezinol
Biochianin A		İzoksanthohumol	Sekoizolarisirezinol
Formononentin			Pinorezinol

Flavonoidler *Leguminoseae, Rutaceae, Primulaceae, Polygonaceae, Salicaceae, Pinaceae, Rosaceae* familyalarına ait bitkilerde diğer familya bitkilerinden daha yüksek oranlarda bulunmaktadır [31]. Yapılan çalışmalarda temel kaynakları meyveler (narenciye, kuşburnu, kayısı, vişne, üzüm, elma, kuş üzümü, yaban mersini vb.), sebzeler (brokoli, soğan, yeşilbiber, domates, ıspanak vb), içecekler (kırmızı şarap, kahve, çay), kahve çekirdeği, soya ürünleri ve baharatlar [32, 33] olan 4000'den fazla flavonoid çeşidi saptanmıştır [34].

Fitoöstrojen kaynağı olarak üzerinde en fazla araştırma yapılanlar flavonoid grubuna dahil olan izoflovanlar ile flavonoid olmayan lignanlardır. Bu nedenle makalede izoflavon ve lignanlar konusuna daha detaylı yer verilmiştir.

Izoflavonlar, en geniş fitoöstrojen grubunu oluştururlar. İlk kez, 1946 yılında Batı Avustralya'da izoflavonca zengin yeraltı üçgülü (*Trifolium subterraneum* L.) ile beslenen koyunların doğurganlığının azalması, laktasyon bozuklukları, cinsiyet organında değişiklikler, kalıcı kısırlık, uterus sarkması gibi sorunların ortaya çıkmasıyla izoflavonların östrojenik etkinliği araştırılmaya başlanmıştır [35]. Izoflavon fitoöstrojenleri yalnızca *Fabaceae* familyasına ait birkaç bitkide bulunmakla birlikte; yonca'da (%0.5-3.5), manj fasulyesinde (3.51 mg/kg), japon sarmaşığı kökünde (0.95 mg/kg; daidzein formunda), kırmızı üçgülde (%1.5-2.5) ve soyada (%0.1-0.5) da belirlenmiştir [17, 36, 37].

Izoflavonlar ve izoflavon bakımından zengin besinler olan; yonca ve üçgülün hayvan beslenmesinde, soya ve manj fasulyesinin insan gıdası olarak tüketimi fazla olmasına rağmen yapılan çalışmalarda kanserlerin, kardiyovasküler hastalıkların, osteoporozun, menopoz sonrası semptomların önlenmesi ve bilişsel fonksiyonun sürdürülmesi gibi olumlu etkileri üzerine odaklanılmış fakat biyolojik mekanizmaları bugüne kadar tatmin edici bir şekilde açıklanamamış, insan epidemiyolojik ve deneysel çalışmaları nispeten sınırlı kalmıştır [38].

Flavonoid olmayan fitoöstrojen grubunun en önemli temsilcisi olan, bitki hücre duvarının yapısında bulunarak lignin oluşumuna katkı sağlayan lignanlar üzerine yapılan ilk çalışma, 1980'li yıllarda yayınlanmıştır [39, 40]. Bitkilerin sekonder metabolizma ürünleri olan lignanlar, bitkinin çevresel stres faktörlerinden korunmasında ve insan beslenmesinde çok önemli işlevlere sahiptir [41]. Kalp hastalıkları, menopoz semptomları, osteoporoz ve göğüs kanseri riskini azalttığı [42] tespit edilen lignanlar, 60'tan fazla bitki familyasından ve köklerinden, rizomdan, odunsu kısımlardan, gövdeden, yapraklardan, meyvelerden, tohumlardan ve reçinelerden izole edilmiştir [43, 44, 45]. Gıdaların lignan içerikleri 2mg/100g'ı geçmemekle [46] birlikte sırasıyla en yüksek lignan kaynakları; 675 µg/g ile keten tohumu, 17.9 µg/g ile mercimek, 8.6 µg/g ile soya fasulyesi ve 6.5 µg/g ile yulaf kepeğidir [47].

Geleneksel Kullanımda Fitoöstrojen Kaynağı Olarak Tüketilen Bazı Bitkiler

Soya (*Glycine max* L.)

Başta Asya ülkeleri olmak üzere yıllardır gıda olarak tüketilmekte olan soya *Fabaceae* familyasının bir üyesidir [48]. Bitkinin tohumları yaklaşık %36-40 protein [49], % 20 yağ, % 35 karbonhidrat ve %5 madensel maddeler içermektedir [50]. Bitkiler içerisinde tohumları en zengin izoflavon kaynağı olarak bilinir. Yapılan çalışmalarda 100 gram soya fasulyesinde izoflavonun en önemli iki bileşeni olan genistein 111 mg ve daidzein 84 mg bulunmuştur [51]. Antik Çinli'ler bitkinin tıbbi olarak kalp, böbrekler, karaciğer, mide ve sindirim sistemi için yararlı olduğunu bildirmiş [52], geleneksel kullanımda siyah tohumlu genotipleri tedavi ile ilişkilendirmişler, üreme bozukluklarında, kuvvet verici, müshil etkili, romatizmaya karşı ve saç uzamasında kullanmışlardır. Tuzla fermente edilen tohumlar, Çin tıbbında oldukça değerlidir ve soğuk algınlığı, baş ağrısı, kanamalı düşük, düşük tedavisi, sinir ve ateş gibi pek çok hastalıkta kullanılmıştır [53]. Uzakdoğu toplumlarında sıkça tüketilen soyanın kolestrolü düşüren, kalp ve damar hastalıklarına karşı koruma sağlayan, kilo kontrolüne yardımcı olan, menopoz sorunlarını ve kanser riskini azaltan bitki olduğu yönünde araştırmalar bulunmaktadır [54]. Yapılan bir çalışmada, soya proteini tüketimi, postmenopozal makak maymunlarında kazein tüketimi ile kıyaslandığında lipid peroksidasyonunda düşüş göstermiş, tavşanlarda aterosklerozi azaltmıştır [55, 56]. Her bir proteininde aktif bileşikler bulunan soyanın, LDL kolestrolü düşürdüğü ve HDL kolesterolünü yükselttiği ileri sürülmektedir [23]. Araştırıcılar; ortalama 47 g soya proteini içeren bir diyetin toplam kolesterolü yaklaşık % 9,3, LDL kolestrolü % 12,9 ve trigliseritleri % 10,5 azalttığı ve HDL kolestrolü % 2 artırdığını bildirilmişlerdir [57, 58].

Menopoz ve perimenopozal kadınlarla yapılan bir klinik çalışmada, günlük soya izoflavon takviyesi ile sistemik arteriyel uyumun düzeldiği gözlemlenmiş [59], menopoz öncesi dönemde soya tüketen kadınlarda meme kanseri riskinin önemli derece azaldığı saptanmıştır [60]. Buna karşın post menopozal dönemde tedavi gören meme kanserli kadınlarda 5 ay, günlük 80 mg soya izoflavon tüketiminin meme ucundan alınan sıvıların salgısını ve hiperplazi gibi iyi huylu tümörlerin büyümesini arttırdığı belirlenirken [61], günde 60 g soya proteini verilen bir çalışmada, ortalama günlük sıcak basma sayısının soya tüketen grupta önemli ölçüde azaldığı belirlenmiştir [62].

Yapılan çalışmalara göre göğüs kanseri, osteopoz ve menopozal semptomlarda soya ürünlerinin tüketim miktarı, süresi, dozu kişiden kişiye ve hastadan hastaya değişkenlik gösterdiği için kıyaslamaların doğru bir değerlendirme olamayacağı [56, 63], soya tüketiminin güvenilirliği ve etkisi için daha fazla çalışmaya ihtiyaç duyulduğu belirtilmektedir [64]. Hatta soya tüketiminde toplumların tüketim alışkanlıklarının bile etkili olabileceği dikkate alınmalıdır.

Meyankökü (*Glycyrrhiza glabra* L.)

Fabacea familyasına ait çok yıllık Avrasya kökenli bir bitkidir. Yunanca'da *Glycyrrhiza* 'tatlı kök' anlamındadır [65]. Tıbbı bitki olarak kullanımına dair en eski kayıt M.Ö 2100 yılına aittir [66]. Çin'de sağlıklı olmayı destekleyici ve detoksifikasyon etkilerinin yanı sıra tatlandırıcı ve lezzet verici ajan olarak binlerce yıldır tüketilmektedir [67]. Tıbbi olarak teskin edici ve balgam söktürücü olup, antioksidan ve antimikrobiyal aktivite gösterir [65]. Ülkemizde harareti gidermek için şerbet formunda Diyarbakır, Mardin, Şanlıurfa bölgesinde bol miktarda tüketilmektedir [68]. Göğüs yumuşatıcı, balgam söktürücü, öksürük kesici, mide hastalıklarında (gastrit gibi) tedavi edici, mukoza koruyucu, yanık ve yara tedavisinde kullanılmaktadır [53, 66, 68]. Kuzey Amerika'nın doğal bitkisi olmamasına rağmen, Kızılderililer tarafından öksürük, astım ve balgam sökmenin [69] yanı sıra kadın hastalıklarında [70] da kullanıldığı belirtilmektedir. Meyankökünün ana bileşeni şekerden 50 kat daha tatlı olan glycyrrhizin'dir [68]. Bitki bu özelliği nedeniyle hiperlipidemi, alerjik iltihap, atopik dermatit, damar tıkanıklığı tedavisinde klinik olarak kullanılmaktadır [71]. Toz halinde eczacılıkta, kıvam ve şekil vermede kullanılır. Kola adı altında hazırlanan alkolsüz içeceklerin bileşiminde bulunur [68].

Meyankökünün östrojenik aktivitesi ilk kez 1950 yılında açıklanmıştır [72]. Meyankökünün kök ekstraktlarında östrojenik aktivite gösteren glabren, glabridin ve isoliquiritigenin, 2',4',4'-üç hidroksi kalkon bulunmuştur. Bir isoflavon olan glabrinin, lipofilik yapısı nedeniyle E_2'ye benzer yeni bir fitoöstrojen olarak kaydedilmiştir [71]. Yapılan bir çalışmada meyan kökü ekstresi, PR (progesteron reseptörü) ve ER (östrojen reseptörü)'nin her ikisinde de zayıf bağlanma eğilimi göstermiştir [73]. Meyan kökü etanol ekstraktı içerisinde yüksek östrojenik aktivite göstermiştir [71]. In vitro ortamda yapılan insan östrojenine bağlı glabridininin etkilerinin incelendiği çalışmada konsatrasyonun artmasıyla östrojene bağlı meme kanseri hücrelerinde iki yönlü olumlu

etki görülmüştür [74]. In vivo sonuçlar meyankökü kökünün iskelet ve kardiyovasküler dokular üzerine olumlu etki yaptığını [74], ayrıca yedi erkekte 4 gün boyunca günlük 7 g meyankökü tüketiminin serum testosteron miktarını ciddi oranda azalttığını ortaya koymuştur [75]. Sıçanlar üzerine yapılan benzer bir çalışmada testosteron üretimi azalmıştır [76].

Bu bilgilere rağmen meyan kökünün fitoöstrojen kaynağı olarak tüketiminin yararı ya da zararı üzerine daha fazla araştırmaya ihtiyaç olduğu belirtilmektedir [64]. Ayrıca tüketim şeklinin, miktarının, yaş ve kişi gruplarına göre değişimlerinin de, yöntemlerinin de belirlenmesi gerekmektedir.

Şerbetçiotu (*Humulus lupulus* L.)

Cannabaceae familyasından, çok yıllık, iki evcikli, tırmanıcı bir bitkidir. Dişi çiçekler bitkiye has acı tadını veren humulon ve lupulon türevleri ile lupulin içermektedir [77]. Geleneksel kullanımda iştah açıcı, idrar artırıcı, terletici, ateş düşürücü ve yatıştırıcı etkileri olduğu bilinir [67, 77]. Kızılderililer tarafından böbrek iltihaplarında, göğüs ve uterus şikayetlerinde yatıştırıcı olarak kullanmıştır [69]. Şerbetçiotu dişi çiçeklerinde östrojenik olarak 8-prenilnaringenin, 6-pirenilnaringenin, ksanthohumol ve izoksanthohumol bileşikleri tanımlanmıştır [78]. Şerbetçiotu'nun östrojenik aktivitesini belirlemek amacıyla yapılan çalışmada; kültür ortamında FSH hormonu taklit edilerek, şerbetçiotu ile estradiol sentezinin yapılması planlanmıştır. Bu amaçla dişi farelerden olgunlaşmamış yumurtalar alınmış, bir kısmına 48 saat boyunca FSH, diğer kısmına 48 saat boyunca Şerbetçiotu'ndan elde edilen preparat verilmiştir. Şerbetçiotu preparatı uygulanan farelerin yumurtalıklarında diğer örnekler gibi estradiol sentezlendiği görülmüştür. Bu etkisi sebebiyle şerbetçiotu'nun dişi çiçeklerinin östrojenik aktivite gösterdiği belirtilmiştir [79, 80]. Sıçan uterusu üzerine yapılan bir başka çalışmada *H. lupulus'*ta bulunan 8-prenilnaringenin bileşiğinin östrojen reseptörlerine bağlanma eğilimi gösterdiği saptanmıştır [80, 81]. Milligan ve ark.(1999)'a göre, alkollü bir içecek olan birada yüksek miktarda bulunan (18ng/mL) 8-prenilnaringenin, şerbetçiotunun fitoöstrojen özelliğinin önemli bir kısmını oluşturmaktadır [80].

Almanya'da kadınların menstrual dönemlerinde yaşadıkları ateş basması, ağrılı adet sancıları ve uzun süren adet dönemi gibi şikâyetler [82, 83, 84] ile östrojenik aktivitelerinin düzenlenmesi için şerbetçiotu kullanımının yararlı olduğu belirtilmiştir [85]. Bununla birlikte, doğal göğüs büyütücü olarak pazarlanan şerbetçiotu içerikli bir

diyet takviyesinin, insandaki biyolojik etkilerini belirlemek üzere yapılan in vivo çalışmada; diyet takviyesi ile uterus düzeyinde östrojenik etkiler yaratma ihtimalinin olmadığı, bu etkinliği göstermek için destekleyici kanıtlara ve yeni çalışmalara ihtiyaç olduğu belirtilmiştir [86, 87].

Keten (*Linum usitatissimum* L.)

Linaceae familyasında yer alan bitki, tohumu ve lifi için kullanılır. En eski bitkilerden biri olan ketenin kelime anlamı "çok kullanışlı"dır [88]. Avrupa Farmakopesi'ne tıbbı bitki olarak dahil edilen, Tropikal ve Ilıman iklim bölgelerinde yetişen ketenin lif kaynağı olarak kullanımına ait ilk bulgular Mısır lahitlerinde yer almaktadır [89]. Geleneksel tedavide, tohumları yangıyı azaltmak, sakinleştirici, soğuk algınlığı, öksürük ve bunlara bağlı ateşi azaltmak için kullanılan keteni [64], Kızılderililer ateş, akciğer hastalıkları, şiddetli soğuk algınlığı ve öksürük için [69] ve yağını da gevşetici olarak kullanmışlardır [88]. Keten, α-linolenik asit gibi çoklu doymamış yağ asitlerini içerdiğinden dolayı yağlı tohum olarak oldukça ilgi görmüştür [64]. Keten tohumu, lignan fitoöstrojenleri bakımından en zengin kaynaklardan biri olup [90] kaliteli fitoöstrojen kaynağı olduğunu gösteren çalışmalar bulunur [91, 92, 93].

Tüm dünyada fitoöstrojen özelliği bilinen keten ile yapılan bir in vivo çalışmada, farelerin beslenme diyetlerine %10 keten tohumu ilavesinin tümör gelişme oranını ve metastazı azalttığı görülmüştür [94]. Bleodon ve Szapary (2004)'e göre, memeli canlılarda beslenme yoluyla %5 keten tohumu alımının tümör oluşumunu azalttığı gözlemlemiştir [95]. Altı hafta süresince yağsız keten tohumu liganları tüketiminin araştırıldığı başka bir çalışmada LDL kolesterol ve total kolesterolde azalma görülmüş, keten tohumu lignanlarının HDL kolesterol üzerine etkisi önemsiz bulunmuştur [96]. Menopoz şikayetleri (ateş basması ve vajina kuruluğu) olan 145 kadına 12 hafta süresince, fitoöstrojen bakımından zengin diyet ürünleri (soya ürünleri ve keten tohumu) tükettirilmesinin menopoz semptomlarında bir azalma sağladığı görülmüştür [97]. Bleodon ve Szapary (2004)'e göre, sahip oldukları biyolojik aktiviteler nedeni ile lignanlar, hormona dayalı kanserlerin önlenmesinde ve tedavisinde geleneksel östrojen tedavilerine alternatif oluşturabilecek takviye gıda kaynağı olarak gösterilmiştir [95].

Adaçayı (*Salvia officinalis* L.)

Salvia L. cinsi *Lamiaceae* (Ballıbabagiller) familyasına bağlı yaklaşık 900 türü içerisinde barındıran dünyanın her yerinde görülebilen bir taksondur [98]. *Salvia* türlerinin tıbbi özellikleri ilk kez Romalı'lar tarafından farkedilmiştir. *Salvia* türleri arasında tıbbi olarak eski çağlardan beri bilinen ve kullanılan *Salvia officinalis*'tir. Adaçayı, Eski Mısırlı'lar tarafından çocuk sahibi olamayan kadınlara tükettirilmiştir [99].

Salvia türlerinin yaprakları geleneksel tıpta önemli bir kullanıma sahiptir [100]. Çok eski yıllardan bu yana halk arasında soğuk algınlığı, öksürük, sinirsel bozukluk, sindirim sorunu, faranjit, ağız içi iltihabı, diş eti iltihabı gibi hastalıkların tedavisinde kullanılmış, ayrıca terlemeyi önleyici ve laktasyonu artırıcı etkilere sahip olduğu belirtilmiştir [101, 102]. Adaçayı uçucu yağında bulunan monoterpenler terlemeyi önlediği için [103] ateş basması şikayeti olan kadınların adaçayı tüketmeleri tavsiye edilmiştir [104]. Bir grup post menopozal dönemdeki kadınla 8 hafta boyunca yapılan çalışmada, adaçayı tabletleri tüketen kadınların gece terlemesi, ateş basması ve diğer menopoz şikayetlerinin kontrol grubuna göre önemli azalmalar gösterdiği, böylece hormon replasman tedavisi alamayan kişiler için alternatif bir tedavi olarak dikkate alınması gerektiği belirtilmiştir [105].

Adaçaynın geleneksel olarak postmenopozal semptomları düzelttiği bilinmesine rağmen, kemik kaybının azalmasını önleyip önlemediği hakkında çok az bilgi vardır. Menopoz ile kemik kaybı yaşayan fareler üzerinde yapılan bir çalışmada, adaçayı uygulamasının kemirgenlerin kemik kaybını önlemede etkili olduğu bulunmuştur. Bu durum adaçayının kemik erimesi tedavisinde kullanılabileceğini göstermekle birlikte tavsiyeler vermeden önce daha fazla klinik çalışmalara ve adaçayı çay ekstraktlarında kimyasal maddenin analizlerine ihtiyaç vardır [106].

Isırgan (*Urtica diodica* L.)

Isırgan, yeryüzünün ılıman bölgelerinde yetişen, *Urticacea* familyasına ait, otsu uzun ömürlü ve çiçekli bir bitkidir [107, 108, 109]. Eskiden beri geleneksel ve modern tıpta kellik tedavisi, egzema gibi cilt hastalıkları [110], osteoporoz [111] tedavisinde kullanılan bu bitki organlarında lignanlar, flavonoidler, steroller, polisakkaritler, lektinler ve yağ asitleri [112], C vitamini ve demir [113] gibi etkili bileşikler bulunur. Isırgan otu Türkiye'de geleneksel olarak; idrar söktürücü, eklem ağrılarını dindirici, akne ve hemoroid tedavisinde kullanılmaktadır [114].

Isırganın erkeklerde üreme fonksiyonlarını etkilediğine dair pek çok çalışma yürütülmüştür. Yapılan bir çalışmada; bitkinin fitoöstrojen etkisine dayanarak ısırgan yaprağı ekstraktlarının kemirgenlerde testosteron seviyesini düşürdüğü, sperm üretimini etkilediği belirlenmiştir [115].

Isırgan kök ekstraktlarının kadınlarda yumurta oluşumu, östrojen ve progesteron hormonlarını artırdığı, üreme üzerinde olumlu etkisi olduğunu saptanmıştır. Fakat bu sonuca rağmen konu hakkında daha fazla çalışma yürütülmesine ihtiyaç duyulmaktadır [116].

Civanperçemi (*Achillea millefolium*)

Asterace familyasının bir üyesi olan bitki ülkemizde civanperçemi, amel otu, akbaş otu ve akbaşlı gibi isimlerle bilinmektedir [117]. Türkiye'de 40 civarında *Achillea* türü yetişmekte ve bu türler özellikle Kuzey ve Doğu Anadolu'da yayılış göstermektedir [118]. Civanperçemi geleneksel kullanımda yara iyileştirici, emenagog (regli kanamasının uyarılması), amenore (regli görülmemesi), anemi, antihelmetik, antiinflamatuar, antiviral, diüretik, dizanteri, diyare, epilepsi, grip, hipertansiyon, histeri, kızamık, kızarıklık, kontraseptif, melankoli, pnömoni, suçiçeği, romatizma, tüberküloz, ülser [119, 120, 121] gibi hastalıkların tedavisinde kullanılmaktadır. Ayrıca; idrar sökücü, iştah arttırıcı özelliklerinin yanı sıra basur tedavisinde etkili olduğu bildirilmiştir [118].

Civanperçemi iyi bir anti-kanser aktiviteye sahiptir. Yapılan çalışmalarda, civanperçeminin fitokimyasal bileşenleri olan flavonoid ve seskiterpenler; fare lösemi hücresi P-388'e, epitel beze uruna, göğüs epitel beze uruna (MCF-7) ve deri epidermoid karsinom (431) hücrelerine karşı etkili bulunmuştur [122]. Bitki türleri üzerinde aktif bileşenlerin belirlenmesine yönelik çalışmalarla doğru bitki materyallerinin seçilmesine ihtiyaç vardır [123].

Maydanoz (*Petrosellium crispum*)

Apiaceae familyasından otsu bir bitkidir [124]. 100g. taze maydanoz % 85 su, % 15 kuru madde içermekte olup, bunun 2,2 g. protein, 0,3 g. yağ, 1,3 g. karbonhidrattır [125]. Fenolik bileşikleri flavonoidler (apigenin, apiin ve 6"-Acetylapiin), esansiyel yağ bileşikleri (Mirsitin ve apiol), kumarinler ve furokumarinlerden oluşan maydanoz; antioksidan, karaciğer koruyucu, sinir sistemi koruyucu, anti diyabetik, analjezik, spazmolitik, immün baskılayıcı, pıhtılaşma karşıtı, anti ülser, müshil, östrojenik, diüretik,

düşük tansiyon, anti bakteriyal ve anti fungal gibi kanıtlanan pek çok kimyasal özelliğe sahip bir bitkidir [126]. Ülkemizde geleneksel olarak idrar ve safra artıcı, adet söktürücü olarak da kullanılmaktadır [127]. Maydanozun topraküstü kısımlarının metanollü ekstraktlarında östrojenik aktivitenin incelendiği çalışmada soya izoflavonlarına eşit östrojenik aktivite belirlenmiştir [128]. Maydanozda pek çok biyoaktif bileşen tespit edilmiştir. Fakat bu bileşiklerin hangisinin farmakolojik aktivitelerden sorumlu olduğuna dair deneylere ve çalışmalara ihtiyaç vardır [126].

Sarımsak (*Allium sativum* L.)

Liliaceae familyasından keskin kokulu bir bitki [129,130,131] olan sarımsak karbonhidratlar (sakkaroz, glikoz), vitaminler (A,B,C) ve kükürtlü bir uçucu yağ içermektedir [132]. Sarımsağa özel koku ve lezzetini veren kükürtlü bir uçucu yağ olan allisin [133], sarımsak dişleri ezildiğinde açığa çıkan insan sağlığı açısından çok önemli bir bileşiktir [134]. Eski dönemlerden beri geleneksel olarak antiseptik, idrar arttırıcı, solucan düşürücü, iştah açıcı, tansiyon düşürücü [132], kalp damar hastalıklarından koruyucu, kolesterolü düşürücü, bakteriyal, viral, mantar enfeksiyonlarına karşı etkili, bağışıklık sistemini güçlendirici, antitümör ve antioksidan özelliklere sahip olduğu [135], bu etkinin taşıdığı allisinden kaynaklandığı bildirilmiştir [136].

Sarımsağın kansere karşı koruyucu etkilerine ilişkin araştırmalar bulunmaktadır [137]. Kanserli farelere sarımsak ekstraktı enjekte edildiğinde tümör hücrelerinin çoğalmasını engellemiş ve doğrudan kanser hücrelerinde mutasyona yol açmıştır [138]. Yapılan araştırmalar sarımsağın, göğüs, özefagus, mide, kolon ve rektum kanserlerine neden olan karsinojenlere karşı, canlı dokularda koruma sağladığını ortaya koymuştur [139, 140]. Erkek fareler üzerinde yapılan bir çalışmada sarımsağın prostat gelişimine sebep olan kanser hücrelerini baskıladığı [141], yüksek tansiyonu düşürücü etkiye sahip olduğu, protein hasarını ve antioksidan enzimlerindeki düşüşü onardığı belirlenmiştir [142]. Sarımsak bileşiklerinin kadınlarda meme, erkeklerde prostat kanserinin önlenmesinde yararlı olduğu ve tiroid kanser hücrelerini baskıladığı, kolon, akciğer ve deri kanseri riskini azalttığı belirtilmektedir [143].

İki gruba ayrılmış koroner kalp hastaları ile yapılan çalışmada üç yıl boyunca hiç sarımsak verilmeyen gruptaki hastaların ölüm oranının sarımsak verilenlerden iki kat fazla olduğu, sarımsak verilenlerde kalp krizi geçirme oranının, tansiyon ve kandaki kolesterol seviyesinin daha düşük olduğu belirlenmiştir [144]. Hipertansiyonlu hastalar üzerine

yapılan bir çalışmada sarımsak tüketiminin kan basıncında azalmaya neden olduğu tespit edilmiştir [145]. Sarımsak tozu ile 12 ay süreli yapılan bir çalışmada 12. ay'ın sonunda LDL-kolestrol seviyesinin erkeklerde 32.9 mg/dL ve kadınlarda 27.3 mg/dL düşüş gösterdiği gözlemlenmiştir [146]. Araştırıcılara göre bu sonuçlara rağmen sarımsağın etkisini kesinleştirmek için deney süresini artırmanın yanı sıra büyük bir istatistik grup ile klinik çalışmalar yaparak daha detaylı veriler elde etmeye ihtiyaç vardır [147].

Fitoöstrojenlerin Bazı hastalıklar Üzerine Etkisi

Kanser ve Tümör

Araştırıcılar tarafından, beslenme ile yüksek miktarda izoflavon ve lignan alımı göğüs kanseri gelişimi riskinin azalması ile ilişkilendirilmiş [148,149,150], Batı toplumlarında göğüs kanseri oranının Asya toplumlarından daha yaygın olmasının sebebi fitoöstrojenlerle daha az beslenmeye bağlanmıştır [151,152]. Yapılan çalışmalar düşük yağ, yüksek soya proteinleri, sebze yağları ve karotenden zengin sebzeler ile beslenmenin menopoz öncesi dönemde kadınlarda göğüs kanseri riskini azalttığını ortaya koymaktadır [24,150]. Prostat kanseri erkekler arasındaki en yaygın kanser türlerinden bir tanesidir ve beslenme ile fitoöstrojen alımının prostat kanseri hücrelerinin büyümesini engellendiğini gösteren pek çok çalışma yürütülmüştür [21, 153, 154, 155, 156, 157, 158].

Kalp Sağlığı

Endüstriyel toplumlarda kadın ölümlerinin yaygın sebebi olarak koroner kalp hastalıkları gösterilmekte, menopozla östrojen kaybı sonucunda koroner kalp hastalıkları riski artmaktadır [58]. Yapılan çalışmalar izoflavon ve lignan fitoöstrojenleri ile beslenmenin kardiyovasküler hastalık riskini azalttığını [56,159,160], günde ortalama 47 g soya proteini alımı sonucunda; total kolestrol düzeyinde %9.3, LDL kolestrolünde %12.9 ve trigliseritte %10.5 oranında azalma olduğunu ortaya koymuştur [57].

Menopozal Semptomlar

Fitoöstrojenler ve menopozal semptomlar üzerine yürütülen pek çok çalışma bulunmaktadır [22, 161, 162, 163, 164]. Ateş basması gibi menopozal semptomlar üzerine hormon replasman tedavisinin faydalı etkileri bulunmuş, ancak bu yöntem göğüs ve endometriyal kanser riski ile de ilişkilendirildiğinden hastalar alternatif tedavi

yöntemlerine başvurmuştur [64, 165]. Batı ülkelerinde menopozal semptomların azaltılması amacı ile yukarıda özetlenen bazı bitkiler kullanılmıştır [22, 73].

Kemik Sağlığı ve Osteoporoz

Osteoporoz kadınlarda genellikle menopozla ilişkilendirilmiş, fitoöstrojenlerin kemik mineral yoğunluğunu etkilediğine dair hayvanlar ve insanlar üzerinde çalışmalar yapılmıştır [56, 166, 167, 168]. Hormon replasman tedavileri ile postmenopozal kadınlarda kemik kaybı önlenebilmektedir. Bununla birlikte yapılan çalışmalarda fitoöstrojenlerin kemik sağlığının korunmasında hormonlardan daha etkili olduğu [169, 170], fitoöstrojenlerin kemikte östrojen reseptörlerine bağlanarak östrojenik etki gösterdiği ve bu şekilde menopozla oluşan kemik yıkımını azalttığı ortaya konmuştur [171, 172].

Algılama ve Hafıza

Araştırmalarda; menopoz döneminde algılama ve hafıza fonksiyonlarının düştüğü belirtilmektedir. Bu yüzden çalışmalar fitoöstrojenler ve algılamanın yanı sıra, algılama ve östrojen replasman tedavisinin ilişkisine yoğunlaşmıştır [168]. Yapılan çalışmalarda fitoöstrojenlerle beslenmenin hafızayı güçlendirdiğine dair kanıtlar bulunmuştur [173, 174]. Buna rağmen soya ürünü olan tofu tüketiminin algılama bozukluğunu artırdığını ortaya koyan aksi yönde sonuçlar sonuçlar mevcuttur [175].

Fitoöstrojenlerin Antiöstrojenik Aktivitesi

Fitoöstrojenlerin aktivitelerinin ortamın östrojen düzeyi ile ilişkili olabileceği ve yüksek östrojenli durumda (menopoz öncesi) antiöstrojenik etki, düşük östrojenli durumda ise (menopoz sonrası) östrojenik etki gösterebilecekleri düşünülmektedir [176, 177]. Menopoz öncesi fitoöstrojen bakımından zengin gıdalarla beslenen kadınlarda; östradiol, progesteron, seks hormon bağlayıcı globülin (SHBG) düzeylerinde azalma, FSH (folikük stimüle eden hormon) ve LH (luteinize edici hormon) da baskılanma gibi endokrin değişiklikler görülmesi fitoöstojenlerin menopoz öncesi dönemde antiöstrojenik aktivitesini doğrular nitelikte bulunmuştur [178].

Fitoöstrojenlerin Yan Etkileri

Fitoöstrojenlerin potansiyeli ve mekanizması tam olarak belirlenemediği için tüketilirken dikkat edilmesi gerekmektedir [179]. Yüksek miktarda üçgül tüketen koyunlarda kısırlık ve üreme bozukluklarının görülmesi [34, 180], esaret altındaki çitalarda, soya fasulyesi ürününden oluşan kedi diyetini tüketirken doğurganlık oranlarında azalma, diyetten çıktıktan sonra normale dönüş yaşanması [181] tartışmalara yol açmıştır. Yapılan bir çalışmada fitoöstrojenlerin erkek farelerde antiöstrojen olarak hareket edip üremeye zarar verdiği ve endokrin sistemini bozduğu [182, 183], yavru sıçanlarda kumestrol tüketiminin testosteron yoğunluğunu baskıladığı, yetişkin sıçanlarda ise anormal üreme davranışlarına sebep olduğu [184] ortaya konmuştur.

Sonuç

Fitoöstrojenler üzerine çalışmalar yıllardır devam etmektedir. Araştırmacılar, soya ağırlıklı beslenen Asya topluluklarında düşük kardiyovasküler hastalıklar, güçlü kemik yapısı, göğüs kanseri oranı ve ateş basmalarının az olmasının nedenini fitoöstrojenlere bağlamışlardır. Fitoöstrojenlerin faydalarını gösterir nitelikte pek çok kanıt olmasına rağmen daha fazla klinik çalışmaya ihtiyaç vardır. Fitoöstrojenlerin güvenli kullanımları için çalışmaların materyal metotları incelendiğinde; kullanılan bitki materyalinin hangi gelişme döneminde, hangi türden, günün hangi saatinde alındığı bilinmemektedir. Kullanılan materyallerin çoğunun içerisindeki fitoöstrojen maddelerin oranları belirtilmediği gibi sinerjik/allelopatik etki gösterebilecek bileşenlere yer verilmemiştir. Fitoöstrojenik bitki kaynağı belirtilirken araştırmada yer alan kişi/hayvan deneklerinin yaşı, cinsiyeti, bulunduğu yaşam ortamı ile bitkinin yetiştiği bölge, hasat ve kurutma koşulları ayrı ayrı ele alınmalıdır. Bu nedenle, tüm çalışmalarda sekonder metabolit olarak tanımlanan fitoöstrojenlerin günün saatine, bitki materyalinin yaşına, lokasyonuna, iklim koşullarına göre değiştiği göz önünde bulundurularak materyal metot kısmında bunlara önemle yer verilmesi gerekmektedir. Böylece bilgilerin bu bölümde detaylandırılması elde edilen verilerin güvenilirliğini artıracak ve bilimsel verilerin geliştirilmesine katkı sağlayacaktır.

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Research Article

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Effects of Potato Virus Y Strains on Local Tomato Genotype "Sazlıca"

Musadik Abdullahi Ahmed[1], Eminur Elci[2*]

ABSTRACT

The aim of this study was to investigate incidence of PVY and the effect of PVY strains on the yield and fruit quality of local tomato genotype "Sazlıca", which is important for local producers and farmers at different stages of development. Symptomatic samples were collected from Sazlıca region to investigate PVY infection incidence and 10 % of samples were found positive by DAS-ELISA analysis. Seedlings were mechanically inoculated with two different PVY strains in three combinations ($PVY^{NW,}$ $PVY^{NTN,}$ and $^{NW+NTN}$) at four different growth stages (7, 14, 21 and 28 days old) for the assessment of PVY strains' effects on plants. PVY susceptible commercial tomato cultivar H2274 was used as control. Among the PVY strains, PVY^{NW} alone has shown the maximum infection rates in the replications and varieties. Moreover, 7 days old inoculated plants have displayed the maximum PVY infections as expected, whereas only one infection was observed in 28 days old plants. There were no symptoms detected on fruits for all replications. In the fruit quality and yield parameters, the highest fruit number, length, width, weight, and brix value were observed on PVY^{NW+NTN} within 21 days plants. PVY infections on Sazlıca tomatoes were proven and PVY^{NW} was found to be the most effective strain for the yield and fruit quality of Sazlıca tomatoes among the tested strains. Based on the data provided by this study, it is thought that it will be possible to develop more effective management methods for PVY virus.

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Introduction

Tomato (*Solanum lycopersicum* L.) plant is a family member of Solanaceae, including more than 3000 species with many economically important plants. Due to their morphological and ecological differences, *Solanum* species can be found on all warm and tropical continents. [1].

It arises from Andean land presently covering in part of Chile, Ecuador, Bolivia, Colombia and Peru. In Turkey, Adana province had the highest tomato production at the beginning of

[1] Niğde Ömer Halisdemir University, Faculty of Agricultural Science and Technologies, Plant Production and Technologies Department Niğde, Turkey
[2] Niğde Ömer Halisdemir University, Faculty of Agricultural Science and Technologies, Plant Production and Technologies Department Nigde, Turkey
*Corresponding Author: Eminur Elci, e-mail: eminur@gmail.com

the 19th century. Therefore tomato production has great contribution in Turkey's economy [2, 3]. In 2017 tomato production was 182.302.395 million tons whereas tomato harvested area was 4.848.384 worldwide [4]. Being the most grown vegetable in the whole world adds up to its the importance. First countries in both fresh and paste tomatoes production are USA, Italy followed by Turkey. In 2017, the production potential of Turkey was of 187,070 tons [5]. There are certain factors with key roles for the advancement of tomato production in Turkey, such as appropriate ecological conditions, income source for growers, and increase in public demand [6]. Comparing the other vegetables and fruits processed, tomato paste furnishes the greatest amount of foreign exchange earned by our country. Tomato processing also creates employment which is a great economical support for agriculture [7]. It also has a significant economic value as frozen food, canned product, pickles, fruit juice, paste, and ketchup.

This economical plant has many threats in nature lowering its agricultural yield and quality. *Potato virus Y* (PVY) is the most harmful virus found in tomato and other *Solanaceous* crops production areas [8]. PVY has a broad host range normally infecting plants of more than nine families, containing 14 genera of the *Solanaceae*, including tomato, pepper, tobacco, and eggplant [9]. This virus spreads with the help of small insects called aphids. Aphids are the most important and hazardous vectors of potato viruses with more than 40 species transmitting PVY in natural conditions [10]. The current classification of PVY isolates are carried out based on primary hosts, symptoms caused in various plants, and serological response to monoclonal antibodies. These isolates have been categorized in three major strains; PVY^N, PVY^O and PVY^C [11]. Strains of PVY includes PVY^N (Tobacco venial necrosis strains), PVY^O (Ordinary stains), and PVY^C (Stipple-streak strain, including potato virus C). The major diseases caused by PVY consist of mild to harsh leaf mottling, leaf-drop streak (PVY^O) with necrosis along the veins of underside the leaflets (PVY^N), and stipple streak (PVY^C) [12]. Making comparison to other Solanaceae crops, tomato appears to be insufficiently selective considering symptoms caused by diverse PVY isolates [13]. PVY^C, PVY^O and PVY^N strains infect tomato plant [14] while PVY^O and PVY^C strains infect pepper only [15]. Severe mosaics, frequently followed by interveinal yellow and whitish spots on potato fruits indicate infections by PVY^N strains. In the past 20 years, two

new PVY variants were identified and assorted as subgroups of PVY^{N} strain, PVY^{NTN} and PVY^{NW} (In North America named $PVY^{N:O}$). PVY^{NTN} is the causal agent of potato tuber necrotic ring spot disease and PVY^{NW} was found in Poland in 1984 [16].

Control of the economic damages caused by this viral pathogen is a burning question in the agricultural practices of 21st century. Moreover, to ensure an effective control strategy of this pathogen there are certain features that pointed out, such as its unique nature as its mode and site of infection, favorable environmental conditions, different biotic and abiotic factors, and age of infection. Infection of PVY in different growth age of tomato hinders the total quality of the products. Hence, this pathogen causes great damage in both small fields and large scale productions areas. As a result of extreme nature and huge losses, PVY is ranked at 5th position in term of worldwide economic damages among crop pathogens [17].

Sazlıca tomato is one of the most consumed tomatoes in Sazlıca town and Niğde province. It is also source of income in Niğde. It is known to be rich in salt compared to other local tomato varieties. There is no study on PVY incidence and its effects on this local important tomato genotype Sazlıca reported up to date. The purpose of this research is to investigate PVY incidence and the effect of PVY infection at different developmental stages (after 7, 14, 21 and 28 days germination) on yield and fruit quality of local tomato genotype *"*Sazlıca" in Sazlıca town and Niğde region.

Materials and Methods

Materials for the study

Plant materials

To evaluate the effects of PVY infection at different growth stages "Sazlıca" genotype (168 plants), along with PVY susceptible H2274 commercial tomato (174 plants) were tested. The seeds of Sazlıca tomato were collected from various commercial companies in Niğde town, while the seeds of H2274 were obtained from the faculty of Agricultural Science particularly the Department of Plant Production and Technologies.

Virus isolates

PVY isolates from *Potato virus* Y^{NW} *and Potato virus* Y^{NTN} strains were used in the experiment. Tobacco plants inoculated with these isolates were obtained from Prof. Dr. Çiğdem Ulubaş Serçe (NOHU).

Field survey

The survey was conducted during the summer growing season especially between June and July in 2018. A total of 50 leaf samples showing suspicious virus symptoms were randomly collected from different fields in Sazlıca town. Each field a number of samples were obtained; Field 1 (7 samples), Field 2 (11), Field 3 (14 samples) and Field 4 (18 samples). The samples were collected by plastic bags and instantly stored in a cooling box and later transferred in to laboratory refrigerator under -80 °C until testing time.

Experimental design

To evaluate the effects of virus infection time at different growth stages on local cultivar Sazlıca tomato, seeds were germinated in plastic pods at greenhouse condition. As a control, commercial tomato variety H2274 was used. Each plot was included fifteen plants with three replications. After 7 days, 14 days, 21 days and 28 days of germination, two virus strains were used for inoculation in three different combinations; PVY^{NW}, PVY^{NTN} and PVY^{NTN+NW} with uninfected controls.

Mechanical inoculation of virus

During the growing stages of tomato plants, the PVY virus strains (PVY^{NW} and PVY^{NTN}) were inoculated on tobacco plants and these plants were later used for inoculation source for tomato plants. Virus inoculation for tomato plants was carried out by mechanical virus inoculation method. Leaf extract was added, PVY inoculation buffer (pH: 7.4) including 0.199 g/l KH_2PO_4, 1.14 g/l Na_2HPO_4, 0.1% Na_2SO_3, and 1% PVP-40 were used. Infected tobacco plant materials were ground in mortal and pestle to macerate the tissue and it was the initiating step in the preparation of plant leaf extract for inoculation. The plants were kept in shade for 24 hr in order to sensitize them for the virus and following day virus inoculating on plants' leaves were performed. At the starting point, carborundum was sprinkled on to the leaves and the virus preparation was rubbed on tomato leaf surface in such a way as to break the surface cells without making too much mechanical damage. The

leaves were rinsed with tap water soon after 2-3 hours of inoculation. Then, inoculated plant leaf samples were collected representing whole plant part. These inoculations were repeated at 7^{th}, 14^{th}, 21^{st}, and 28^{th} days.

Double antibody sandwich enzyme-linked immune sorbent assay (DAS-ELISA)

PVY presence was tested on the samples collected from the department's greenhouse and the survey samples collected from Sazlıca town area by using DAS-ELISA, according to the instructions of antisera's manufacturer (Bioreba AG, Switzerland) corresponding to the PVY polyclonal antisera [18]. Before samples analyzed, each sample was transferred into new 2 ml tube and added with extraction buffer; 200 µl of sample juice and 200 µl of extraction. In the first step of DAS-ELISA, coating was done with 40 µl of IgG in 40 ml of coating buffer and 200 µl were added to each well. The plates were covered tightly and incubated 30 °C for 4 hours. Then plates were washed with washing buffer three times. Each well was covered with 200 µl of sample and placed in a humid box and left at 4 °C for overnight. Following day plates were washed as in step one, then 200 µl of 40 µl of enzyme conjugate in 40 ml of conjugate buffer solution was added into each well. Plates were placed in humid box and incubated at 30 ℃ for 5 hours. Then the plates were washed as described previously and 200 µl of dissolved pNPP (Para-nitro phenyl-phosphate) at 0.04 mg in 40 ml of substrate buffer was added into each well along with positive and negative control samples and extraction buffer were added on each well. This procedure was repeated in triplicates. The plates were incubated at room temperature (20-25 ℃) for 2 hours. ELISA result was visually observed based on yellow color development in the plates and then measured at 405 nm on Biotek EL800 ELISA reader.

Calculation of PVY infection rate

In order to determine the percentage of PVY in inoculated tomato plants, the following formula were used:

$$\textbf{\% PVY incidence} = \frac{\textbf{Tomato samples confirmed positive by ELISA}}{\textbf{Total tomato samples tested}} \times \mathbf{100}$$

Yield and fruit quality parameters

Ripen fruits from each sampling in the three different replication (stages) were collected with clean plastic bags by hand and were stored in a refrigerator at +4 °C until the study was conducted. These different parameters such as fruit length (FL), fruit width (FW^{th}) and fruit weight were observed and fruits were also measured for their soluble-solid content (Brix content) by using A. KRÜSS Optronic GmbH, AR-2008.

Data analysis

The samples were also compared to present the variance among replications and strains. The fruit quality parameters were analyzed by using post hoc, Duncan, the software IBM SPSS statistical 25 version.

After all analysis, all the infected samples were autoclaved for the control of contamination.

Results

Field survey

During the survey, different virus symptoms were observed in the tomato fields at Sazlıca town. The tomato plants showing viral infection symptoms were collected and tested by DAS-ELISA. The symptoms we focused on include leaf rolling, necrotic, yellow leaf curl, and stunting. Five samples in the fields located in Sazlıca have shown positive results. The highest number of PVY positive reactions was observed with the samples collected from Field 1; 14%, in comparison to the samples collected from Field 4, Field 2, and Field 3 with 11%, 9%, and 7% infection rates respectively from Sazlıca town. The average percentage of PVY positive samples of the fields from Sazlıca town were 10%.

Mechanical inoculation

The symptoms appearance on tomato leaves varied according to inoculation time, PVY strain, plant age, and environmental conditions. PVY symptoms usually became visible four to five weeks after inoculation (Figure 1). Samples were collected from all inoculated plants along with controls, and were tested for the presence of PVY using DAS-ELISA. The main symptoms observed on the plants during infection development are shown in Figure 1.

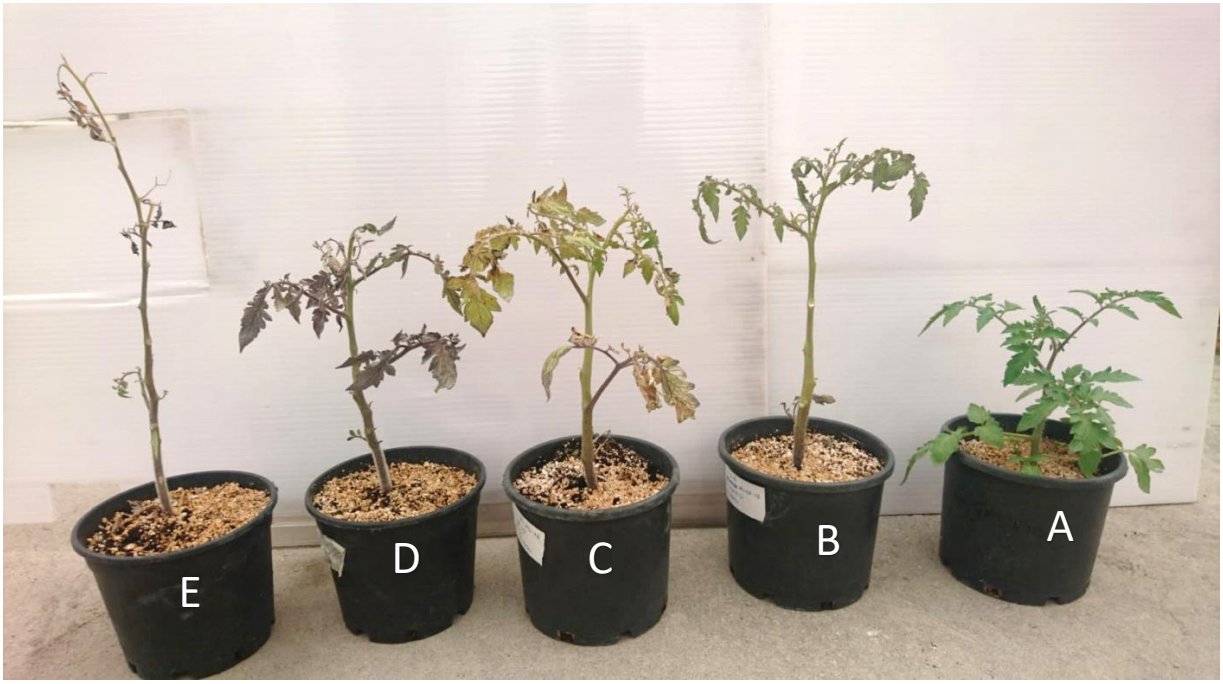

Fig 1 (**A**) Healthy plant, (**B**) leaf rolling and yellowing (**C**) Leaf yellowing and terminal leaves dying (**D**) severe yellowing and slight dark brown color (**E**) Dark brown color and all leaves dying.

DAS-ELISA

The samples of local genotype "Sazlıca" and H2274 plants inoculated by $PVY^{NW,}$ PVY^{NTN}, and mixed of PVY^{NW}+PVY^{NTN} have shown negative and positive reactions. All control plants were negative. PVY have been identified in 112 out of 392 inoculated samples. The result of DAS-ELISA was visually observed with yellow color development in the wells of ELISA plates. On seven days inoculated plants, the maximum ELISA positive result was recorded for H2274 with mixed PVY strains (PVY^{NW+} $PVY^{NTN)}$ (84.62%), followed by Sazlıca with PVY^{NW} strain (72.72%). In fourteen days inoculated plants, the maximum ELISA positive results were achieved by Sazlıca with the PVY^{NW} strain (53.53%). In twenty-one days inoculated plants, the maximum ELISA positive results were obtained by H2274 with PVY^{NTN} (46.67%). In twenty-eight days inoculated plants, only one positive sample was found and it was Sazlıca with PVY^{NW}. The details of all ELISA positive results are also summarized in Table 1.

Table 1 The details of ELISA result in Sazlıca and H2274 varieties and PVY strains in 7 days, 14 days, 21 days and 28 days inoculations' during the current study

Variety	Inoculation date	Total # of tested samples	Total # of infected samples	PVY strains	Infection rate (%)
Sazlıca	7 days	33	8	PVY^{NW}	72.72%
		(11 plants per strain)	7	PVY^{NTN}	63.63%
			5	PVY^{NW+NTN}	45.45%
H2274		39	9	PVY^{NW}	69.23%
		(13 plants per strain)	5	PVY^{NTN}	38.46%
			11	PVY^{NW+NTN}	84.62%
Sazlıca	14 days	45	8	PVY^{NW}	53.33%
		(15 plants per strain)	7	PVY^{NTN}	46.67%
			7	PVY^{NW+NTN}	46.67%
H2274		45	8	PVY^{NW}	53.33%
		(15 plants per strain)	7	PVY^{NTN}	46.67%
			6	PVY^{NW+NTN}	40%
Sazlıca	21 days	45	3	PVY^{NW}	20%
		(15 plants per strain)	2	PVY^{NTN}	13.33%
			2	PVY^{NW+NTN}	13.33%
H2274		45	7	PVY^{NW}	46.67%
		(15 plants per strain)	6	PVY^{NTN}	40%
			3	PVY^{NW+NTN}	20%
Sazlıca	28 days	45	1	PVY^{NW}	6.67%
		(15 plants per strain)	0	PVY^{NTN}	0%
			0	PVY^{NW+NTN}	0%
H2274		45	0	PVY^{NW}	0%
		(15 plants per strain)	0	PVY^{NTN}	0%
			0	PVY^{NW+NTN}	0%

Yield and fruit quality parameters

The effect of PVY at different growth stages on yield of local tomato genotype “Sazlıca” and H2274 variety on fruit length, weight, width and brix were analyzed. The results obtained were analyzed with variance test in post hoc, Duncan, SPSS and are summarized in Table 2.

Table 2 Fruit quality parameters results of 7 days, 14 days and 21 days inoculated plants (the average of infected plants along with control plants)

Tomato /virus strain	7 days inoculated plants				14 days inoculated plants				21 days inoculated plants			
	Fruit length (cm)	Fruit weight (g)	Fruit width (cm)	Brix (%)	Fruit length (cm)	Fruit weight (g)	Fruit width (cm)	Brix (%)	Fruit length (cm)	Fruit weight (g)	Fruit width (cm)	Brix (%)
H2274 PVY^{NTN}	3.64	27.08	4.03	5.91	3.55	17.83	2.32	3.50	3.62	21.45	3.75	4.50
H2274 PVY^{NW}	4.03	34.29	3.30	5.24	2.47	28.58	3.57	4.58	1.83	12.01	1.50	1.73
H2274 PVY^{NTN+NW}	3.78	18.80	4.00	5.42	3.35	27.64	4.05	5.73	1.16	6.11	1.33	1.83
Sazlıca PVY^{NTN}	1.77	17.21	2.39	3.27	3.34	27.32	3.56	4.48	3.66	20.16	4.16	4.26
Sazlıca PVY^{NW}	2.31	18.11	2.90	3.94	2.97	20.55	2.94	4.56	1.50	13.70	1.66	1.90
Sazlıca $PVY^{NTN+}PVY^{NW}$	2.40	26.36	3.70	5.16	3.88	26.38	4.27	5.58	4.16	38.39	4.50	6.16
H2274 Control	4.35	39.44	4.00	6.32	4.70	45.38	4.50	6.26	4.10	48.20	4.30	6.60
Sazlıca Control	4.60	30.60	3.85	5.56	4.50	42.66	3.90	6.30	4.80	47.31	4.10	6.50

Fruit quality parameters of seven, 14 and 21 days old inoculated plants

The H2274 variety with PVY^{NW}+PVY^{NTN} and PVY^{NW} in 7 days and H2274 variety with PVY^{NW+NTN} and PVY^{NW} in 21 days inoculated plants obtained the maximum fruit length of 5.5 cm while Sazlıca genotype with PVY^{NTN} and PVY^{NW+NTN} in 14 days inoculated plants obtained the highest height of 5 cm. Both H2274 variety with PVY^{NTN} in 14 days inoculated plants and Sazlıca genotype with PVY^{NW+NTN} in 21 days inoculated plants are recorded with the maximum fruit with of 5.5 cm. The H2274 variety with PVY^{NW} in 7 days inoculated plants achieved the maximum fruit weight of 55.80 g/plant. On the other side Sazlıca genotype with PVY^{NTN} in 14 days inoculated plants showed maximum fruit weight as 51.08 g/plant. The H2274 with PVY^{NTN} in 14 days inoculated plants are observed with maximum fruit brix as 8.1%, while Sazlıca genotype with PVY^{NW} in 14 days inoculated plants showed maximum fruit brix of 6.8% (Table 2).

Discussion

PVY is the most economically important disease problem in tomato plants in many places through the world. This virus is responsible for decrease in yield and quality and causes serious diseases in cultivated hosts, such as tomato, potato, tobacco, and pepper. A total of 392 tomato plant from local genotype “Sazlıca” and PVY susceptible H2274 commercial tomato varieties were mechanically inoculated with PVY strains at different times (7 days, 14 days, 21 days and 28 days). After 6-8 weeks, samples were collected from inoculated plants along with 50 survey samples and tested by DAS-ELISA. The positive result was variable based on the replications, varieties and effect of PVY infection. Based on the survey studies in Sazlıca region, some virus specific symptoms were observed on tomato plants and the results indicate PVY incidence in the region. Due to the high amount of potato and other *Solanecous* plant production in the area, PVY could be easily transmitted to Sazlıca tomato genotype as well. Therefore it has crucial importance to successfully to prevent from and control PVY infections in Sazlıca tomatoes.

Effects of PVY strains on Sazlıca tomatoes were tested with mechanical inoculations under greenhouse conditions. Although the varieties (Sazlıca and H2274), have shown a close positive result, H2274 is found to be more susceptible to PVY infection than Sazlıca

genotype. In seven days inoculated H2274 plant, 25 out of 39 samples has shown positive, whereas Sazlıca have indicated 20 out of 33 samples as positive. In fourteen days inoculated plants, 22 out of 45 Sazlıca samples were positive, while H2274 has shown 21 positive samples out of 45 samples. In twenty-one days inoculated plants, H2274 has shown 16 positive samples out of 45 samples whereas Sazlıca has displayed 7 samples out of 45 samples. In twenty-eight days inoculated plants, Sazlıca has only 1 positive sample out of 45 samples and H2274 has not indicated any positive sample in all samples.

*Among the PVY strains, PVY^{NW} has shown the highest positive reaction in the varieties and the replications except PVY^{NW+NTN} in seven days inoculated plants. PVY^{NW+NTN} showed the highest infection rate in seven days inoculated plants. The infection of PVY^{NW} in Sazlıca seven days inoculated plants was 72.72% compared PVY^{NTN} (63.63%) and PVY^{NW+NTN} (45.45%). While in H2274 seven days inoculated plants, PVY^{NW+NTN} (84.62%) has shown the highest infection rate compared with PVY^{NW} (69.23%) and PVY^{NTN} (38.46%). In Sazlıca fourteen days inoculated plants; PVY^{NW} was the highest percentage which was 53.33%, whereas PVY^{NTN} and PVY^{NW+NTN} had reached a similar percentage which was 46.67%. On the other hand, H2274 fourteen days inoculated plants, PVY^{NW} had the maximum infection rate which was 53.33% compared with PVY^{NTN} (46.67%) and PVY^{NW+NTN} (40%). In Sazlıca twenty-one days inoculated plants, PVY^{NW} had the maximum infection rate which was 20%, whereas the two other strains (PVY^{NTN} and PVY^{NW+NTN}) had similar infection rate (13.33%). No symptoms were observed on fruits for all replications. Therefore, it can be concluded that the PVY^{NW} strain has an importance in management of PVY in Sazlıca tomatoes.

In H2274 twenty-one days inoculated plants, the PVY^{NW} was also had the highest infection rate which was 46.67% compared with PVY^{NTN} (40%) and PVY^{NW+NTN} (20%). In the last inoculated plants, which were after twenty-eight days, only Sazlıca had shown one positive sample and this was from the PVY^{NW} (6.67%) strain while the other strains did not indicate any positive reaction.

The age of the plant is an important factor that takes part the susceptibility of the plants to the viruses. As the age of the plant was older, the effects of the virus were lower. The plants that inoculated after seven days were the most susceptible ones to virus when compared to

other plants inoculated later. The plants inoculated after twenty-eight days were affected by other factors and did not show infection for the virus except one sample which indicates the importance of plant age for infections.

The most previous studies focused on the resistance of plant to the virus and the virus vector. The symptoms of PVY infection differ with varieties, PVY strain, plant age and environmental conditions. A biological assay resulted that cv. Agria is more susceptible to $PVY^{N\text{-}Wi}$ than to PVY^{NTN}, whereas cv. Charlotte is susceptible to both strains. The biological (inoculation) assay also displayed that the expression of symptoms on varieties is strain-dependent. These shows stress the main role of the resistance profile of varieties to explain the balance of the PVY strains in potato crops [19]. The occurrence of PVY on potato plants were investigated and samples were collected from main tomato growing fields in west-bank Palestine by utilizing serological, biological and molecular methods. In DAS-ELISA method, the occurrence of PVY virus was identified at an average of 15.29% and also confirmed by RT-PCR analysis and bioassay test [20]. Researchers studied the distribution and percentage of PVY strains (PVY^{N}, PVY^{O}, PVY^{C}) along with other viruses such as PLRV and PVS and seed tubers were used for sowing materials in the significant potato producing provinces in Turkey [21]. The symptoms induced by single or mixed infection were observed under field conditions. At first, virus-specific polyclonal antibodies were used to analyze a total of 880 leaf samples and almost 83 samples were detected the presence of PVY infection of the first result were re-analyzed by utilizing PVY^{O}, PVY^{N}, PVY^{C}-virus-specific monoclonal antibodies. The ELISA result showed seed potato tubers utilized for the planting materials were infected with the percentage of PVY (17.7%), PLRV (14.2%), PVS (4.6%) and PVX (11.8%). Another group found that all PVY isolates infecting tomato and pepper as a positive for the normal strains of PVY^{O} both ELISA and RT-PCR whereas PVY isolates infecting potato have more heterogeneous and consisted of PVY^{N}, PVY^{NTN} and $PVY^{N\ Wilga}$ strains and some cases mixed infection shown [22]. However, our research had several limitations which are fruit quality parameters and have showed a non-significant in statistical analyzing. In our findings, the PVY susceptible commercial H2274 tomato variety is more susceptible to PVY^{NW} than to PVY^{NTN} and PVY^{NW+NTN}. It is reported that infection rate was higher for plants inoculated at pre-

flowering relative to those inoculated at the post-flowering and the replication different for mechanical inoculation, the interaction of strain and genotype was not statistically significant [23]. Mature-plant resistance can also inhibit PVY^{N} infections but plants need to be physiologically old at the time of highest infection pressure in the late season. Therefore, the use of chatted seed linked with planting as early as possible and early haulm destruction could together be a helpful part of an approach to manage PVY^{N} [24]. The current study also showed us that the infection rate was higher to the plants inoculated seven days when compared to the plant inoculated later and this indicates the importance of plant age for virus infection.

In the fruit quality parameters, among the three replications (seven days, fourteen days and twenty-one days), varieties and strains, the highest fruit length is achieved by Sazlıca PVY^{NW+NTN} which is recorded for 4.16 cm in twenty-one days inoculated plants. The highest fruit weight is reached by Sazlıca PVY^{NW+NTN} which is recorded for 38.29 g/plant in 21 days inoculated plants whereas the minimum fruit is obtained by seven days inoculated plants. On fruit width, the maximum fruit width among the replications, tomato varieties and strains are obtained by Sazlıca PVY^{NW+NTN} with the maximum average of 4.5 cm in twenty-one days inoculated plants. For fruit total soluble solid content (brix), Sazlıca PVY^{NW+NTN} has the maximum average of 6.16% in twenty-one days inoculated plants when compared with other replications. After seven days inoculated plants yielded smaller and produced less tomato compared to 14 days and 21 days plants. In general fruit brix, the fourteen days inoculated plants obtained the maximum fruit brix with the number of 6.8 % (Sazlıca) and 8.1 % (H2274) when compared the seven days and twenty-one days inoculated plants. On the other hand, control plant all of them are closer and have shown a higher percentage when compared with infected plants. Only one infection was observed on 28 days inoculated plants even for repetitions. The findings also showed us that the survey samples have infections and at least one positive sample was found in each field and this indicates how PVY exists in Sazlıca town area. The twenty-eight days inoculated plants also had not produced any infections due to age and physiological conditions of plants. The mechanical inoculation methods were more effective than virus vector transmissions because of the virus was directly transmitted to plants through leaves with high

concentrations. This study is a base for future study of the effect of PVY strains at different growth stages on yield and fruit quality of local genotype in Turkey particularly Sazlıca town/Niğde region.

Conclusion

Tomato is one of the most important vegetable grown worldwide and is now the fourth most saleable fresh-market vegetable after potatoes, lettuce, and onions. Turkey ranks as the 4th biggest producers of tomatoes around the world. Infection of PVY in different strains and growth ages of tomato hinders the total quality-based production of tomato. The current study has importance for knowing the effects of PVY strains on local tomato genotype Sazlıca and this research can be used as starting point for effect of PVY strains on tomato at different growth stages (after 7,14,21 and 28 days). The farmers in Sazlıca area are recommended to remove host plants to minimize or eliminate virus inoculum sources and they should take necessary precautions to prevent from aphids spread which is the most dangerous vector of PVY in early stages of tomato plantlets to decrease the PVY incidence. The farmers are also recommended to plant susceptible crops away from each other, to use certified seeds, to select resistant varieties and to avoid mechanical transmissions.

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Research Article

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Bioactivities of *Hypericum perforatum* L. and *Equisetum arvense* L. fractions obtained with different solvents

Adem Necip[1*], Mesut Isik[1]

ABSTRACT

In this study, anticholinesterase and antioxidant effects of HPL aqueous extracts (HPLAE), HPL methanol extracts (HPLME), EAL aqueous extracts (EALAE) and EAL methanol extracts (EALME) obtained from *Hypericum perforatum* L. (HPL) and *Equisetum arvense* L. (EAL) were investigated. The HPLME fraction on acetylcholinesterase (AChE) showed an inhibitory effect, while others showed no inhibitory effect. Antioxidant activities of different fractions of HPL and EAL were determined using different in vitro methods including Fe^{3+}- Fe^{2+} reduction capacity, $ABTS^{\cdot+}$ radical scavenging capacity, $DPPH^{\cdot}$ free radical reduction capacity, and CUPRAC methods. In the study, the fractions were compared with the standard antioxidant BHT, BHA, and Trolox. The fractions obtained from these plants have 52% radical scavenger activity close to standards, and moderate metal reduction activity. As a result, different fractions of these medicinal plants used to treat many diseases caused by oxidative stress have varying bioactivities.

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Introduction

Acetylcholinesterase (AChE), important for neurodegenerative diseases, is found at erythrocytes, serum, and cholinergic brain synapses. AChE inhibitors, which inhibit or slow the hydrolysis of acetylcholine (ACh), play an important role in the treatment of many diseases including Alzheimer's disease (AD), myasthenia gravis, and ataxia [1-3]. Furthermore, the AChE inhibitors used in the symptomatic treatment of AD are known to be effective in eliminating the neurotoxic effect of β-amyloid (Aß) on disease development, protecting cells from oxidative damage and producing cellular antioxidants [4-6]. In light of this information, it is thought that the formation of oxidative damage with an excessive increase of AChE activity may be due to the formation of free radicals. The free radicals have an atomic or molecular structure containing unincorporated electrons. The structures, which easily exchange electrons with different molecules, are called " reactive oxygen species (ROS)". Oxidative stress occurs with excessive increase

[1] Department of Pharmacy Services, Vocational School of Health Services, Harran University, 63300, Şanlıurfa, Turkey
*Corresponding Author: Adem Necip ademnecip@gmail.com

of ROS. This causes significant biomolecular damage in organisms [7]. Antioxidants have importance for reducing oxidative stress. Antioxidants have recently become one of the topics investigated by scientists due to their ability to scavenging free radicals. Today, it has been reported that many synthetic antioxidants are used especially in food additives and they negatively affect human health because they are not natural [8]. Phenolic compounds have been reported to be beneficial to health due to their antioxidant properties [9-10]. Many plant species used medically today are known to have rich phenolic content.

Hypericum species are extremely important plants that have been popularly used because of their various pharmacological effects. *Hypericum perforatum* L (HPL), a species of Hypericum known in Turkey as yellow cantaron and blood grass, is commonly known as "Saint John's Wort" around the world. It has been classified as a natural source of food sweeteners (Class 5) by the Council of Europe. Moreover, it has been reported that the plant can be used in the treatment of moderate depression. Furthermore, the studies have reported that HPL can be as effective as antidepressant used in traditional medical treatment [11,12]. It has been reported by the German Ministry of Health that HPL extract is used for the treatment of psychovegetative disorders, depressive disorders, anxiety and/or agitation situations and adjustment disorders [13]. In previous studies, HPL extracts have been reported to show a significant scavenging capacity for free radicals produced by the xanthine oxidase / xanthine system [14]. It has been reported in the literature that the important pharmacological effects of the plant originate from naftodiantron compounds (hypericin, psodohipericin etc.), fluoroglusinols (hyperforin, adhiperforin etc.), flavonoids (hyperositis, routine, quercetin etc.), biflavones (biapigenin, amentoflavones) phenolic acids, (ferulic acid, caffeic acid, etc.), proanthocyanidins, and essential oils [15]. *Equisetum arvense* L (EAL) is considered to be a living fossil that has been used for medicinal purposes since time immemorial and has been protected in many countries [16,17]. In traditional medicine, it has been used in urinary and prostatic diseases, symptoms of the urinary system, repairing lung tissue, lung tuberculosis, hormonal or metabolic edema, rheumatism, and wounds [18]. EAL contains caffeic acid derivatives, saponins, flavonoids, silica, and alkaloids, which have anti-inflammatory and antioxidant effects. EAL has various pharmacological properties as it contains various secondary metabolites such as phenolics, phytosterols, alkaloids, minerals [19-23]. It is

important to know the bioactivities of medicinal plants traditionally used to reduce oxidative stress caused by free radicals. In this study, anticholinesterase and antioxidant effects of HPL aqueous extracts (HPLAE), HPL methanol extracts (HPLME), EAL aqueous extracts (EALAE) and EAL methanol extracts (EALE) obtained from *Hypericum perforatum* L. (HPL) and *Equisetum arvense* L. (EAL) were investigated.

Materials and Methods

Determination of antioxidant activity

Ferric cyanide (Fe^{3+}) reducing antioxidant test (FRAP) was performed by modifying the method reported by Oyaizu (1986). When ferric ions (Fe^{3+}) are reduced to ferrous ions (Fe^{2+}) at 700 nm, the complex is formed and is a method of spectrophotometric measurement of this complex. Reduction capacity (Cu^{2+}) for Cupric ions was determined by the cupric ions reduction assay (CUPRAC) in previous studies [24-26].

DPPH and ABTS radical scavenging activity

DPPH scavenging activities of methanol and water fractions of HPL and EAL were determined according to the method performed by Blois (1958). In the method, the stable DPPH radical is removed by the free radical removal activity of the sample. Extract of 10, 20 and 40 µg/mL was prepared from the samples then the volume with ethanol was adjusted to 1 mL. Then the prepared DPPH solution (1 mL, 0.1 m) was added and left in the dark for 30 minutes. DPPH removal activity of the sample after incubation was measured spectrophotometrically [1,27]. In this method, a sample is added to a pre-prepared ABTS· solution and after 30 minutes, the remaining cationic ABTS radical was measured spectrophotometrically at 734 nm [28]. Then, 1 mL of cationic ABTS radical solution was added to the sample at different concentrations. Absorbance was determined 30 minutes after mixing and for each concentration, radical removal percentage and IC_{50} values were calculated [29].

Determination of enzyme activity

The inhibitory effects on AChE of different HPL and EAL fractions were tested by Ellman's spectrophotometric method [30]. Reaction solution containing 50 µl AChE ($5.32x10^{-3}$ U), 100 µl of Tris–HCl solution (1 M, pH 8.0) and 50 µl 5,5'-dithio-bis(2-nitro-benzoic)acid compound (DTNB) was mixed and incubated at 30 °C for 15 minutes. Then, the reaction was started by adding 50 µl acetylthiocholine iodide (AChI) that was used as a substrate, and was performed spectrophotometric measurement at 412 nm [31].

Result and Discussion

Inhibition of acetylcholinesterase (AChE) that hydrolyzes acetylcholine (ACh), is a basic approach in the symptomatic treatment of diseases such as ataxia, Alzheimer's disease (AD), myasthenia gravis, senile and dementia. Inhibition of AChE is important for increasing ACh levels in the synaptic cavity [1,6,32]. The use of AChE inhibitors such as galantamine, rivastigmine, and donepezil used for the treatment of AD in recent years has been limited due to side effects such as hepatotoxicity, abdominal pain, novelization, nausea, vomiting, and diarrhea. Therefore, it is important to provide potential source of AChE inhibitors from plants that are abundant in nature [33,34]. In a study on the inhibitory effect of different extracts on AChE (*in vitro*), the results showed that methanolic fractions had a more active effect than water fractions. The IC_{50} values obtained for methanolic plant fractions included Nardostachys jatamansi (rhizome), Tinospora cordifolia (stem), Withania somnifera (root), Ficus religiosa (stem bark) Embelia ribes (Root) and Semecarpus anacardium (stem bark) were found in the range of 16.74-73.69 μg/ml [35]. In another study, inhibitory effects on AChE and BChE of rosmarinic acid were investigated. Rosmarinic acid was found to have an 85.8% inhibition effect on AChE at 1.0 mg / mL [36]. In our results, *Hypericum Perforatum* L. methanol fraction (HPLME) showed inhibitory effect on AChE with IC_{50} values of 0.262 ± 0.03 mg/ml, while no inhibitory effect was observed in others (**Table 1**). *Hypericum Perforatum* L. water extract (HPLAE) showed no significant AChE inhibitory potential. The inhibition effect of methanol extract is consistent with the higher inhibitory potential in methanolic extracts of plants in previous studies.

Hypericum species often have good antioxidant effects due to the large number of different phenolic compounds they contain. According to the results of a study in which the free radical scavenging effect of *H. perforatum* was investigated in vitro, the antioxidant effect of the extract obtained was found to be directly proportional to concentration. Plant extract is strongly hydroxyl and superoxide anion- scavenging and prevents lipid peroxidation [37]. Plant fractions may have the potential to control or prevent the formation of reactive oxygen species (ROS) due to their volatile compound and phenolic content [38]. Phenolic compounds that can readily give hydrogen from hydroxyl groups along the aromatic ring prevent the harmful effects of ROS and free

radical oxidation [39]. The antioxidant/anticholinesterase activities and total phenolic content of HPLA, HPLME, EALAE, and EALME are shown in Table 1 in this study.

Table 1 The anticholinesterase and radical removal activity of HPL and EAL fractions in different concentrations

Extracts	DPPH[b] [0.2 mg/ml]	ABTS[b] [0.2 mg/ml]	Total phenolic content (μg GAE mg^{-1} extract)	AChE IC_{50} (mg/mL)
EALAE	5.12 ± 0.3	4.63 ± 0.4	32.27 ± 3.6	inactive
EALME	52.41 ± 4.2	30.65 ± 2.8	30. 3 ± 3.9	inactive
HPLAE	48.64 ± 4.8	60.74 ± 4.6	16.04 ± 1.2	inactive
HPLME	25.44 ± 2.6	6.04 ± 0.4	21.46 ± 2.3	0.262 ± 0.03
BHT[a]	52.54 ± 5.5	58.06 ± 5.3	-	-
BHA[a]	80.59 ± 6.7	93.62 ± 6.2	-	-
Trolox[a]	90.56 ± 6.4	89.99 ± 5.9	-	-

Data mean ± standard deviation,

[a]standard antioxidant

[b]The percent (%) of ABTS and DPPH radical scavenging activity

The study by Dragana D et al. reported that the total phenolic compound was 79.52 ± 3.97 mg/g for EALME and was 25.4 ± 1.19 mg / g for EALAE [40]. In the study by Takeshi Nagai et al., head and stem parts of *Equisetum arvense* were studied and the results reported 12.8 mg/g in water extract for head part, 12.3 mg/g in ethanol extract, 7.98 mg / g in water extract for trunk part, and 23.9 mg / g in ethanol extract for head part [41]. The study by Annamaria Pallag et al. reported a total phenolic amount for EALME as 82.63 ± 0.06 mg / GAE and total flavonoid amount as 71.23 ± 4.33 mg / QE [42]. In addition, in FRAP, DPPH and CUPRAC assay reported results as 84.160 ± 0.078 μm Trolox equivalent/g, 87.30 ± 0.039, 49.2± 0.104 μm Trolox equivalent / G, respectively. The study by Bruno A Silva et al. identified 7 different fractions. *Hypericum perforatum* ethanol extract reported free radical-scavenger activity (IC_{50}) value of 21 lg dwb/ml [43]. In this study, metal reduction capacity was examined as FRAP and CUPRAC. When the

results of metal reduction capacity at 0.2 mg/mL were examined, from large to small HPLAE, EALME, HPLME and EALAE were found. From these extracts, HPLAE and EALME metal reduction capacity was found to be higher than HPLME and EALAE (Fig. 1a-b).

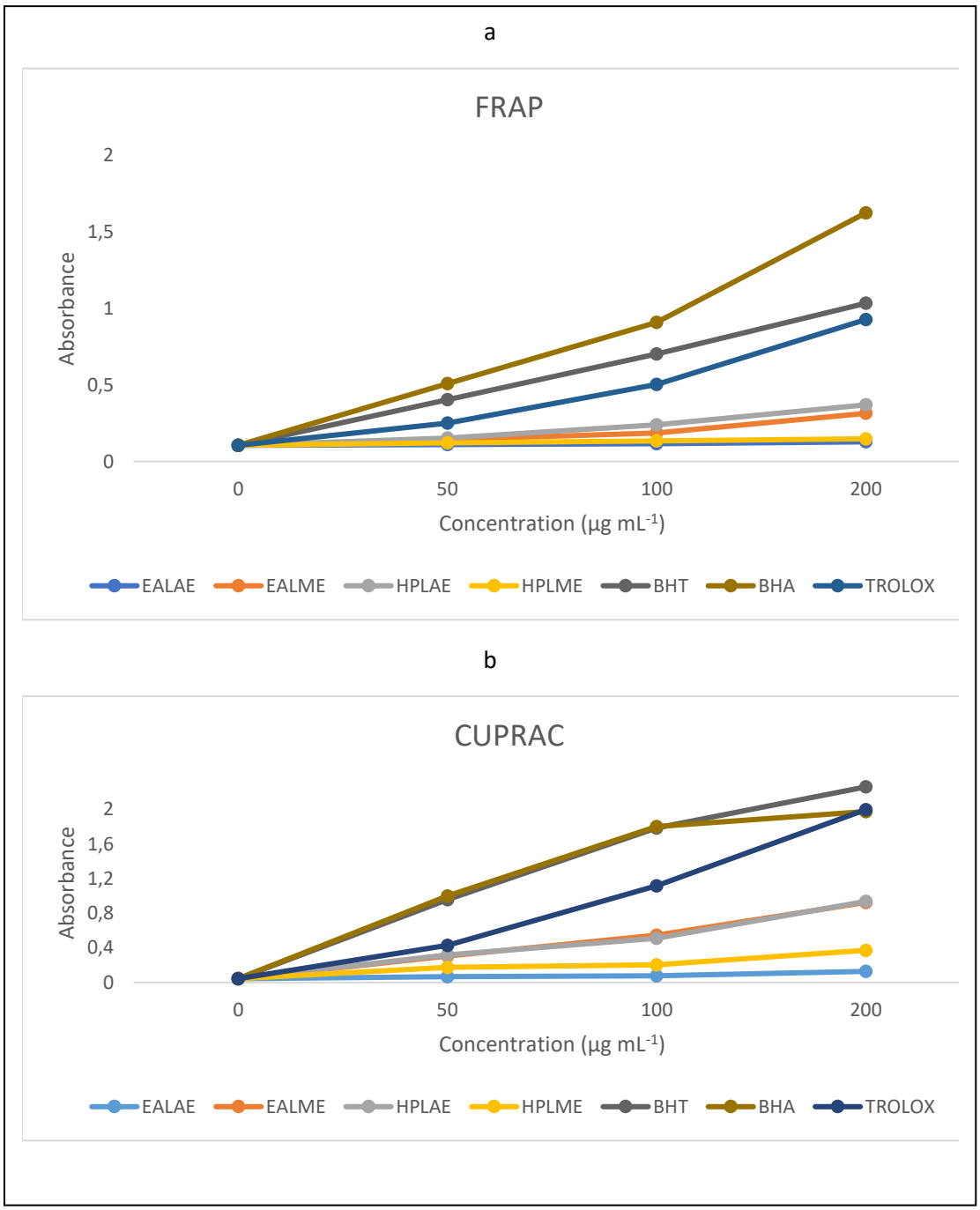

Fig 1 Metal-reducing capacity with FRAP (a) and CUPRAC (b) assays of HPL and EAL fractions in different concentrations

DPPH and ABTS are well-known radical scavenging methods for measuring antioxidant activity. In the present study, EALME showed DPPH radical scavenging of about 52%, HPLAE 49%, HPLME 26%. HPLAE showed ABTS radical scavenging activity of approximately 61% and EALME 31% (Table 1). Total phenolic compound quantities were also found to be approximately 32 (µg GAE mg^{-1} extract) for EALAE, 31 (µg GAE mg^{-1} extract) for EALME, 21 (µg GAE mg^{-1} extract) for HPLME, and 16 (µg GAE mg-1 extract) for HPLAE. The study by Dragana D et al. reported the total phenolic content for EALAE as 25.4 ± 1.19 mg/g. In this study, the total phenolic content for EALAE was found to be 32.269 ± 3.6 µg GAE mg^{-1} extract. The results of our study and previous studies are consistent and supportive of each other. DPPH is a useful reagent for investigating the free radical scavenging effect of phenolic compounds [44]. The reduction of DPPH absorption is an indication of the capacity of extracts to free radicals scavenging. The best free radical scavenging activity was reported for EALME. The lowest activity was observed for the EALAE fraction. The fractions have shown radical scavenging activity close to standard antioxidants. Reactive oxygen species (ROS) are known to damage central nervous systems [45]. For this reason, antioxidants have important radical removal activities to eliminate these harmful effects. As a result, HPLAE and EALME have high antioxidant activities. The incorporation of certain HPL and EAL fractions into food products or pharmaceutical preparations is important in terms of the health-beneficial antioxidant content. It appears that the use of *H. perforatum* fractions does not cause any significant side effects evident in most consumers [46].

As it is known in the literature, it is important to determine the bioactivity of natural products recently. It has been found that some of the methanol and water fractions of HPL and EAL have radical scavenging and anticholinergic effects. HPL and EAL are thought to be used in the treatment of many diseases that develop oxidative stress due to this bioactivity property.

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Research Article

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Alpha S1-Casein Gene Polymorphısm in Nigerian Balami Sheep Breed Indigenous to Mubi

Ismaila Yada Sudi[1,4*], Mohammed Shuaibu[2], Malachi Albert Tizhe[3], Augustine Clement[2]

ABSTRACT

The study was conducted on five Balami sheep breed to determine and characterize the alpha S1 casein gene. Five blood samples were collected from different balami intravenously using sterile needle and syringe. The blood samples were placed in labelled tubes containing ethylene diamine tetraacetic acid (EDTA). The blood samples were transported in on ice cold chain container to the laboratory for analysis. Quick-DNA Miniprep TM kit used for DNA extraction and amplified using forward and reverse primers (CSN1S1F 5'-ACCCCTCAGGTACCCTAAGAAA-3' and CSN1S1R 5'-GTTTATCCCCCACACTGCATTC-3'). The gene was sequenced and blasted against the NCBI database. Single nucleotide polymorphism analysis was performed to ascertain the variations. Multiple sequence alignment and phylogenetic analysis within and with the reference sequence was conducted online bioinformatics tools. Result from the analysis reveals that, the extracted DNA were found on chromosome 6, intron 16 and exon 17. The Balami breeds of sheep showed total number of polymorphic and monomorphic site of 68 and 600 respectively, and percentage of polymorphism of 10.18%. The Balami breed showed one amino acid substitution and genetic variation within breeds. Complete molecular characterization, genotyping and determination of allele frequency of alpha S1 gene in Balami breed of sheep indigenous to Nigeria and its variations is recommended for further research.

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Introduction

The population of sheep in Nigeria is estimated at 33.9 million representing 3.1% of the world's total [1]. Sheep milk production started with the beginning of domestication [2] and production in 2008 represented 4.92%, 0.02%, 1.70%, and 1.44% of milk produced in Africa, America, Asia and Europe, respectively (FAO, 2010). Ovine are better suited than bovine to environmental conditions prevalent in sub-Saharan Africa [4, 5, 6], they are

[1] Department of Biochemistry, Faculty of Science, Adamawa State University, Mubi, Nigeria.
[2] Department of Animal Production, Faculty of Agriculture, Adamawa State University, Mubi, Nigeria.
[3] Department of Animal Physiology, School of Agricultural Technology, Federal Polytechnic Mubi, Nigeria.
[4] North East Zonal Biotechnology Centre, University of Maiduguri, Nigeria.
Corresponding author's Email: yada280@gmail.com H/P: +2348160787132

extensively held as “the cows of the small holder” [7], to provide home supply and self-sufficiency for families, to avoid starving and malnutrition especially in high-quality protein [4, 6, 8]. Ovine milk and meat are widely consumed without inhibition as they thrived well with little supplements on browse and pasture.

Ovine milk product such as cheese and yoghurt can provide a profitable alternative to cow milk products owing to their specific taste, texture, natural and healthy image [9] and ovine milk contains higher level of total solids and major nutrient than goat or cow, especially of average protein and fat [10]. This result in higher cheese yield by approximately 15% in sheep compared to 10% in bovine, as cheese curd constitute mostly fat and casein [7, 11]. Whereas mineral contents of ovine milk are comparable with caprine mostly higher than that in bovine milk [10].

Casein is the main proteins in ovine (MPOM); present in colloidal solution and form 76 - 83% of total milk proteins in ovine [10, 12]. Casein play a nutritive function as a source of amino acids, calcium and phosphorus [13]. There are four casein fractions; namely αs_1-(CSN1S1), αs_2- (CSN1S2), β-(CSN2) and κ- casein CN; CSN3). They are differentiated according to their homology of primary structure [14] and are tightly linked within a 250 kb cluster [15, 16, 17] on ovine chromosome six (OAR6) [18].

The research on determining the relationship between the presence of genetic marker and production traits of animals is being conducted for many years now. In livestock farming the emphasis was put on milk protein genes. With the increasing population in the country the demand for milk proteins through sustainable animal agriculture is increasing. There is vigorous research for efficient production system that will supplement nutrition. Therefore, maintaining genetic variation is very important to avoid the loss of breeds by farmers and consumers. There are numerous breeds of sheep that are extinct and others classified at high risk of loss. There is need for characterization, though studying the genetic polymorphism of milk proteins have raised considerable research interest in caprine and bovine species, there are little description of casein gene polymorphism of ovine milk of native sheep breeds of Nigeria.

Casein genetic polymorphism of milk proteins are of importance as association to quantitative and qualitative parameter in milk proteins and used in breeding strategies and

in the dairy industry. They have effects on quantitative traits and technology properties of milk, it has been shown that ovine genetic polymorphisms affect the physicochemical properties of milk hence, there is need for in-depth knowledge of the genetic polymorphism of indigenous ovine milk proteins for the improvement of the quality of ovine milk for its contribution to the Nigerian dairy industry. It is in the light of this that present work is prompted to study alpha casein gene polymorphism in Nigerian indigenous Balami breed of sheep in Mubi area of Adamawa State, Nigeria.

Materials and Methods

Location and Size

The study was conducted in Mubi which is the second largest town in Adamawa State of Nigeria and covers an area of about 600 Square Km. The town lies about 260 Km north of Yola, the state capital. Mubi metropolitan area situated between Latitude 10 º 05' N/ 10 º 30' N and Longitude 13 º10' E/ 13 º30'E. The town is centrally located on the border line between Mubi-North L.G.A and Mubi-South L.G.A [19].

Metrological data

Mubi has a tropical climate. In winter, there is much less rainfall than in summer. According to Koppen and Gieger, this climate is classified as Aw. In Mubi, the average annual temperature is 25 ºC. About 935 mm of precipitation falls annually. The driest month is January, with 0 mm of rainfall. Most precipitation falls in August, with an average of 258 mm. The warmest month of the year is April, with an asverage temperature of 29.3ºC. In August, the average temperature is 23.4 ºC, which is the lowest for the whole year. The difference in precipitation between the dust month and the wettest month is 258 mm. the average temperatures vary during the year by 5.9 ºC [20].

Experimental Animals and Blood Collection

Blood samples were randomly collected from five adult female ovine (Balami breed of Sheep) in Mubi, Adamawa State. Blood samples were collected through the jugular vein, using a needle and syringe (5 ml) and preserved in EDTA in a tube. All the samples were conveyed to the laboratory in an ice park.

Casein Gene Extraction

The casein gene (DNA) extraction was carried out using Quick DNA^{TM} MicroPrep Kit from Zymo Research according to manufacturer's instruction. 400 µl of Genomic Lysis Buffer was added to 100 µl of blood to make 4:1 volume, and mixed completely by vortexing for about 4-6 seconds and was left to stand for about 5-10 minutes at room temperature. The mixture was transferred to a Zymo- spin^{TM} ll column in a collection tube and was centrifuged at 10,000 × g for one minute, the collection tube was discarded with the flow through.

The Zymo- spin^{TM} ll column was transferred to a new collection tube, 200 µl of DNA pre - wash buffer added to the spin column and centrifuge at 100,000 × g for one minute. Again, 500 µl of g-DNA wash buffer was added to the spin column and centrifuged at 10, 00 × g for one minute.

The spin column was transferred to a clean microcentrifuge tube and 50 µl DNA for elution. Elution buffer was added to the spin column and incubated at room temperature for about 2-5 minute and thereafter, centrifuged at top speed for 30 seconds to elute the DNA. The eluted DNA was immediately used for molecular based application.

All genomic DNA was checked on 1% agarose gel electrophoresis and all amplicons on 1.5% agarose gel electrophoresis. The gel was stained with ethidium bromide and visualized under UV light transilluminator. For a 10 cm × 10 cm minigel cast, 1% agaros gel was prepared by dissolving 0.5g of agarose in 50cm of 1 × TAE (Tris Acetate EDTA) buffer, while 1.5% agarose gel for the amplicons was prepared by dissolving 0.75 g of agarose in 5 ml of 1×TAE. The mixture of agarose and buffer was swilled gently to ensure complete dissolution. The colloidal solution formed was heated in the microwave oven for 1-3 minute or till a clear solution was obtained.

The gel was allowed to cool to about 50°C (gel should not solidify) under running tap. Precaution was taken to prevent water from the running tap from splashing into gel. Ethidium bromide was mixed with DNA samples and loaded in the wells created by the comb in addition to loading dye. Genomic DNA samples was prepared for loading into the well by mixing 4 µl of the extracted DNA sample with 1 µl of the 5× loading dye. 5'-ACCCCTCAGGTACCCTAAGAAA-3' and CSN1S1R 5'-

GTTTATCCCCCACACTGCATTC-3') were designed from reference genomic sequence NC-019463.2. The primers spans intron 16 – exon 17 – intron 17 of casein alpha S1 gene on oar_v17 genomic sequence assembly.

A 20 µl reaction consisting of 4 µl of 5× Solisbiodyne master mix. 0.6 µl each of the forward and reverse primers, 12.8µl of nuclease free water and 2µl DNA template was made. The cycling condition were as follows: initial denaturation at 95°C for 3 minute, denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 40 seconds and final extension at 72 °C for 5 minutes. PCR products was run on 1% agarose, viewed and photographed using UV light according to manufacturer's instruction.

DNA Sequence Analysis

The sequencing was carried out at Xcelri Genomics, India, according to Sanger [21] using F 5'-ACCCCTCAGGTACCCTAAGAAA-3' and R 5'-GTTTATCCCCCACACTGCATTC-3 primers. Sequence was blasted against the database in NCBI and multiple sequence alignment using Multalin [22], Clustal W and trimming of sequence on BioEdit [23]. Phylogeny construction in MEGA X using Nei's genetic distance was used generated in Genalex 6.503 [24, 25], and Muscle phylogenetic Neighbour-joining tree 3.8 [26].

Results and Discussion

Sequence Analysis

The DNA isolated sequences of were presented (Fig. 1-4). The highest number of nucleotide sequence (714) with Balami A sheep breed (Fig. 1), and lowest (651) in Balami D (Fig. 4). This result is not consistent with the findings of Rumunno et al. [27] who observed the gene CSN1S1 encoding αs_2 to have the length of 18438 nucleotide and divided in to 19 exons ranging from 24 to 266 nucleotide, the observed differences may be due to differences of class of casein, exon, breed and geographical location.

>CSN1S1- Balami-1
AAGAAATCCCGGTGAATACTCCAGAGTAGAGGCATTTAAAGATTAGCAAAAGTGCAAGGAGGATAAAGTTGAATTTTTAC
TTTGGAGAGAAAATTTTCCTCCTATTTTCATCAGAATGATTATCACTTATCAAATAGATAGCACTGCTCCACATGTTCCTGA
GTAATGGATTAACATTAGCCATATATCAGAAAGTTATCATCAATTTTCCATTTCAGAAAATTCTAGCATAGGACACTAAGC
ATTTAAGTGTCATGCTTATTCTAAATTTATTTTTATATTATGTTTTCTTCTATTTCCCTTGATACGGCAGCAATATGCAGTCA
TTTAAATGAACTTACCACCACAGTGGCATAGTAATCTTTCCACTGTTCTCAGAGCCAATGGGATTAGGGATGTCAGAGAAT
GAGGGGGCATCAGTGTATTGTGTGCCTAGTGGAAGGTAATACCAGGCACCAGATGGATAGGCGTCCAGCTGGTAGAATTG
TCTGAAAAGCTAGAGAGAAAGACCATTGAAAAAGCGACAGTGTATGAGAAATCAGTTTTGAATCTCTGAAAATGAATCAT

TGCATTCATTTCAGACATGGCTATTCGCATCACAAGAGATGTTTACTCTGTGAGGAAAACAGAGAAACCAAACTCTTCCCT
ATGGTAAATCACAGCAAGGAAATTTTGGAAAGGATTTTTCTTAGGGGACCTGAGGGGGGGGAAAAC

Fig 1 Nucleotide sequence of Balami sheep breed A

>CSN1S1-Balami-2
CAATTTCCCTTACTGTGATTTACCATAGGGAAGAGTTTAGGTTTCTCTAGTTTTCCTCACAGAGTAAACATCTCTTGTGATG
CGAATAGCCATGTCTGAAATGAATGCAATGATTCATTTTCAGAGATTCAAAACTGATTTCTCATACACTGTTGCTTTTTCAA
TGGTCTTTCTCTCTAGCTTTTCAGACATTCTACCAGCTGGACGCCTATCCATCTGGTGCCTGGTATTACCTTCCACTAGGCA
CACAATACACTGATGCCCCCTCATTCTCTGACATCCCTAATCCCATTGGCTCTGAGAACAGTGGAAAGACTACTATGCCAC
TGTGGTGGTAAGTTCATTTAAATGACTGCATATTGCTGCCGTATCAAGGGAAATAGAAGAAAACATAATATAAAAATAAA
TTTAGAATAAGCATGACACTTAAATGCTTAGTGTCCTATGCTAGAATTTTCTGAAATGGAAAATTGATGATAACTTTCTGA
TATATGGCTAATGTTAATCCATTACTCAGGAACATGTGGAGCAGTGCTATCTATTTGATAAGTGATAATCATTCTGATGAA
AATAGGAGGAAAATTTTCTCTCCAAAGTAAAAATTCAACTTTATCCTCCTTGCACTTTTGCTAATCTTTAAATGCCTTTCTT
TGGATTATACCCATGATATACATTAGAATGCATGAGGGGGGAAAAAAAAACCAAAA

Fig 2 Nucleotide sequence of Balami sheep breed B

>CSN1S1-Balami-3
GCAAGGGGGGGGGGGAGCAAAACAAAGGGAAGAGTTTGGTTTCTCTGTTTTCCTCACAGAGTAAACATCTCTTGTGATGC
GAATAGCCATGTCTGAAATGAATGCAATGATTCATTTTCAGAGATTCAAAACTGATTTCTCATACACTGTTGCTTTTTCAAT
GGTCTTTCTCTCTAGCTTTTCAGACAATTCTACCAGCTGGACGCCTATCCATCTGGTGCCTGGTATTACCTTCCACTAGGCA
CACAATACACTGATGCCCCCTCATTCTCTGACATCCCTAATCCCATTGGCTCTGAGAACAGTGGAAAGACTACTATGCCAC
TGTGGTGGTAAGTTCATTTAAATGACTGCATATTGCTGCCGTATCAAGGGAAATAGAAGAAAACATAATATAAAAATAAA
TTTAGAATAAGCATGACACTTAAATGCTTAGTGTCCTATGCTAGAATTTTCTGAAATGGAAAATTGATGATAACTTTCTGA
TATATGGCTAATGTTAATCCATTACTCAGGAACATGTGGAGCAGTGCTATCTATTTGATAAGTGATAATCATTCTGATGAA
AATAGGAGGAAAATTTTCTCTCCAAAGTAAAAATTCAACTTTATCCTCCTTGCACTTTTGCTAATCTTTAAATGCCTTTCTT
TGGATTATACCCATGATATACATTAGAATGCAGTAGGGGGGGAGAAAAAAACAAAAT

Fig 3 Nucleotide sequence of Balami sheep breed C

>CSN1S1-Balami-4
ATCCTTACTGTGATTTACCATAGGGAAGAGTTTGGTTTCTCTGTTTTCCTCACAGAGTAAACATCTCTTGTGATGCGAATAG
CCATGTCTGAAATGAATGCAATGATTCATTTTCAGAGATTCAAAACTGATTTCTCATACACTGTTGCTTTTTCAATGGTCTT
TCTCTCTAGCTTTTCAGACAATTCTACCAGCTGGACGCCTATCCATCTGGTGCCTGGTATTACCTTCCACTAGGCACACAAT
ACACTGATGCCCCCTCATTCTCTGACATCCCTAATCCCATTGGCTCTGAGAACAGTGGAAAGATTACTATGCCACTGTGGT
GGTAAGTTCATTTAAATGACTGCATATTGCTGCCGTATCAAGGGAAATAGAAGAAAACATAATATAAAAATAAATTTAGA
ATAAGCATGACACTTAAATGCTTAGTGTCCTATGCTAGAATTTTCTGAAATGGAAAATTGATGATAACTTTCTGATATATG
GCTAATGTTAATCCATTACTCAGGAACATGTGGAGCAGTGCTATCTATTTGATAAGTGATAATCATTCTGATGAAAATAGG
AGGAAAATTTTCTCTCCAAAGTAAAAATTCAACTTTATCCTCCTTGCACTTTTGCTAATCTTTAAATGCCTTTCTTTGGATTA
TACCCATGATATACATTAGAATGCATGGGGGGGAAAAAAAAAAAAAAAA

Fig 4 Nucleotide sequence of Balami sheep breed D

However, consensus was found at various positions, at position 75-78 (TTT), 217-220(TTT), 515- 518 (GAA), among many other positions within the alignment (Fig. 5). Many consensus were found with respect to the reference, at position 1477-1480 (CTC), 15037-150170(TCT), 15187-15190 (ATT) as observed (Fig. 6). Phylogenetic tree within Balami sheep breed shows that 3A and 3C is genetically distance away from 3B and 3D (7), while phylogenetic tree of Balami sheep with reference showed 3A are genetically closer to the reference, 3C and 3B at the same distance to reference and 3A, 3D distantly away from reference, 3A, 3B, 3D are at the same distance respectively. Multiple sequence alignment within all breeds, showed consensus at position 79-83 (TTT), 97 -100 (AAA), 501-503 (TTT) among many consensus observed through multiple sequence alignment. It was observed that, the nucleotide sequence variation among Balami sheep within 154 bp of

intron 16 and exon 17, the highest number of nucleotide sequence (714) in Balami A and lowest (651) in Balami D nucleotide positions when compared to reference gene NC_040257.1, while the variation in G, A, C, and T, was confirmed by using multiple sequence alignment. The polymorphic sites and frequency of polymorphism confirmed the variation and similarity in the multiple sequence alignment, where amino acids substitution and polymorphism were identified within the open reading frame of the CSN1S1 gene as compared with the reference sequence (DNA sequence: 94714744- 94715051). This result is similar to the finding of Calvo et al., [28] who observed 61 polymorphism in Assaf sheep breed on exon 17.

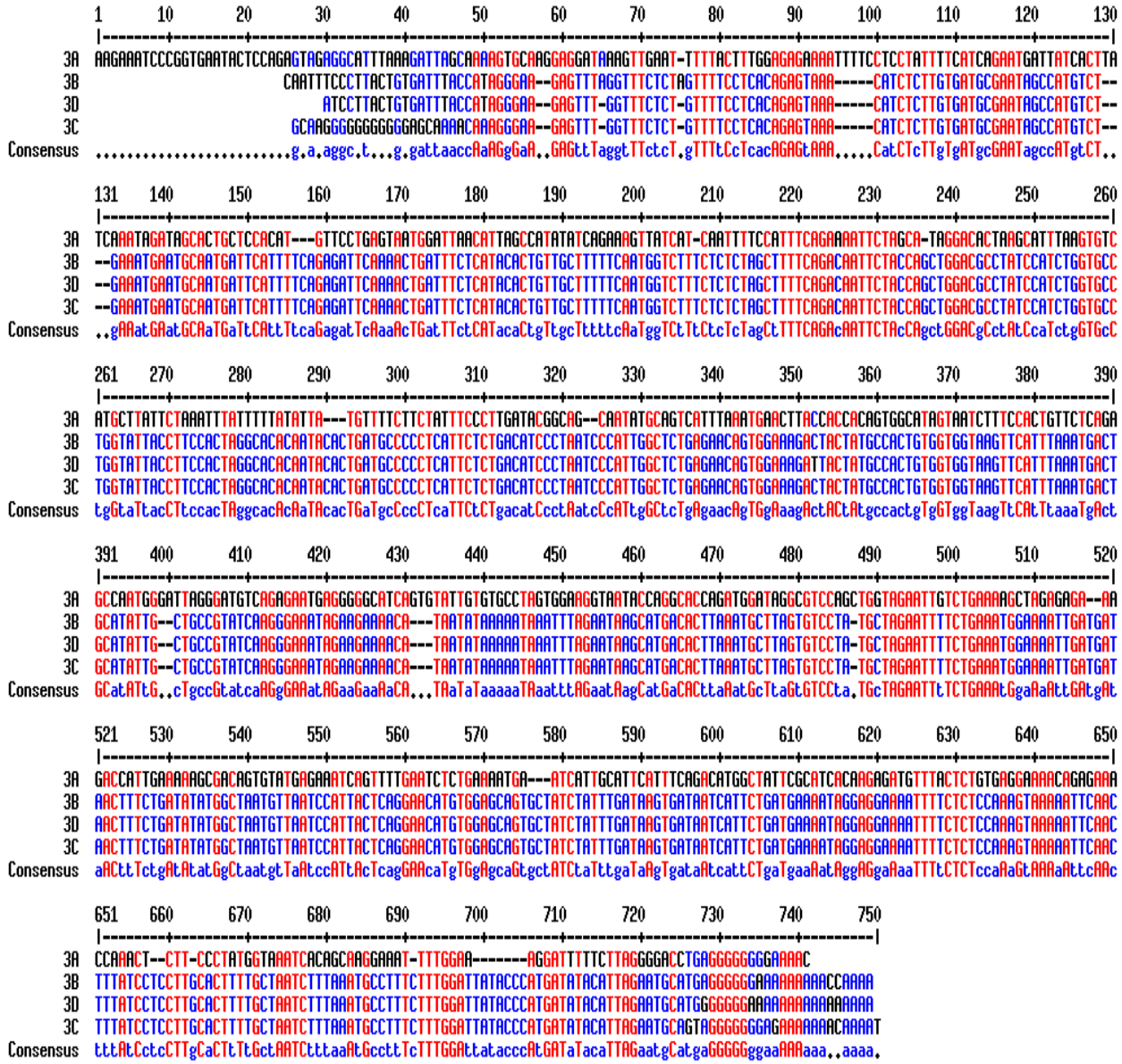

Fig 5 Multiple sequence alignment of nucleotide sequences within Balami sheep breed. Note: 3A =Nucleotide sequence for Balami sheep 1, 3B =Nucleotide sequence for Balami sheep 2, 3D= Nucleotide sequence for Balami sheep 4, and 3C= Nucleotide sequence for Balami sheep 3

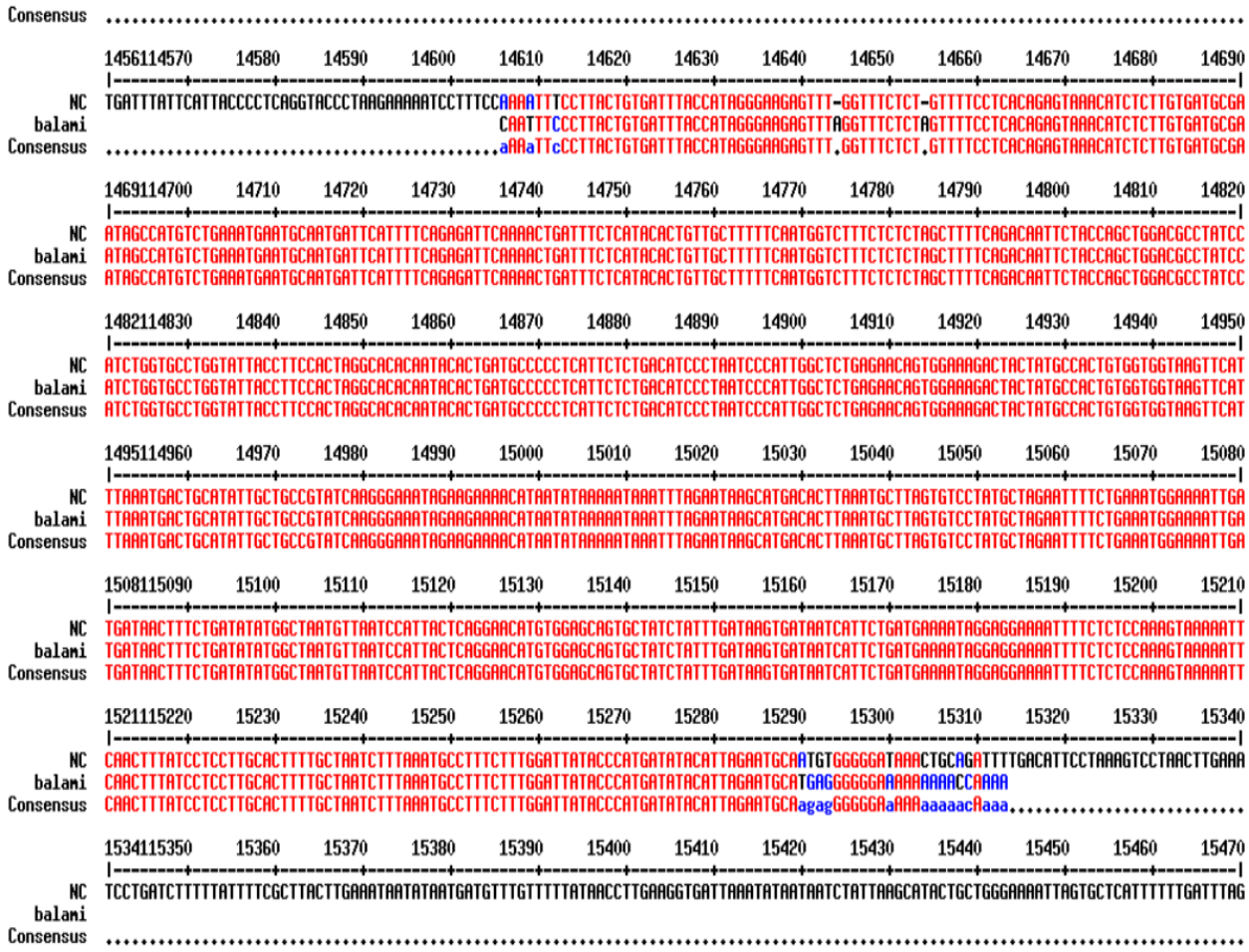

Fig 6 Multiple sequence alignment of nucleotide sequence of Balami sheep with reference sequence. Note: NC= Nucleotide sequence for reference NC-040257.1, balami= Nucleotide sequence for Balami sheep

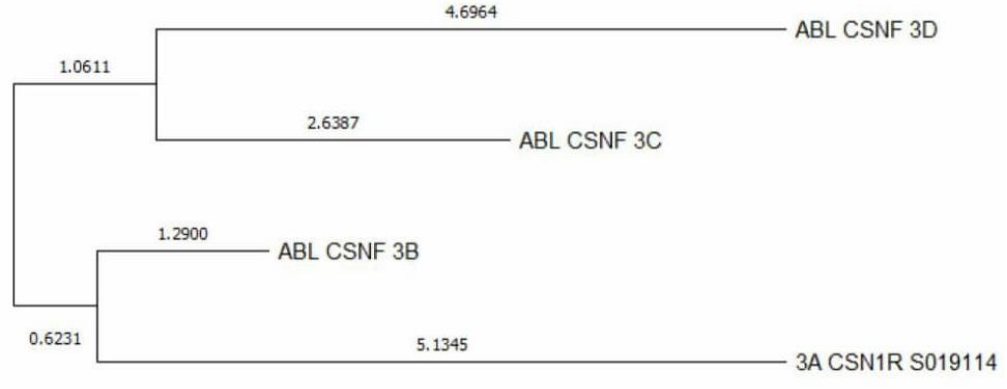

Fig 7 Phylogenetic tree within Balami sheep breeds. Note: 3A CSNIR S019114 = Balami sheep 1, ABL CSNF 3B = Balami sheep 2, ABL CSNF 3C = Balami sheep 3, and ABL CSNF 3D = Balami sheep 4.

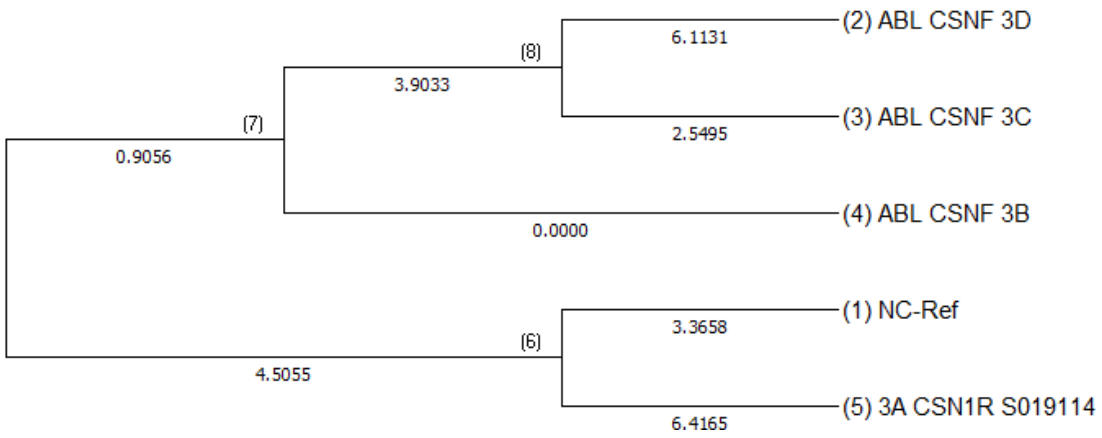

Fig 8 Phylogenetic tree of Balami sheep with reference gene. Note: 3A CSNIR S019114 = Balami sheep 1, ABL CSNF 3B = Balami sheep 2, ABL CSNF 3C = Balami sheep 3, ABL CSNF 3D = Balami sheep 4, and NC-Ref. = Reference sequence.

However, Balami sheep breed showed percentage polymorphism of 5.09%, and the number of polymorphic and monomorphic site of 24 and 644 respectively compared with NC-040257.1 and showed only one amino acid substitution compared with reference.

Although, Chessa et. al., [29] found and reported that amino exchanged at position 200 of αs_2 Asn>lys was observed in bovine, amino acid exchange in Balami was observed at position 201 Thr> Ile with high frequency of 0.733. This difference could be as a result of species different as well as difference of class of casein gene one in Balami sheep breeds indicated high genetic variation within breeds which is very important for breeds adaptability, production and long term survival. All the amino acid exchanged were caused by single nucleotide polymorphism, this is not consistent with the work of Giambra et al. [30]. Balami sheep is genetically distant in repect to the refrence gene (NC_040257.1) by 0.031, and was also in consonance with the phylogenetic analysis and multiple sequenced alignment.

Conclusion

All the balami sheep breeds showed total number of polymorphic and monomorphic site of 68 and 600 respectively, and percentage of polymorphism of 10.18% and the same number of site. Amino acid substitution in Ouda sheep breed was higher (7) than Yankasa sheep breed (4), and Balami sheep breed shown the lowest amino acid substitution of 1, it showed

variation exist within and between breeds these is very important for species long term survival. High frequency of 0.733 was observed at position 201 in all the breeds, showed amino acid exchanged on exon 17 position 183Met>Val with frequency of 0.12 to 0.26 this difference could be as a result of differences in targeted segments on the exon as well as the position of the exchanged. Balami sheep breeds were genetically closer compared to Ouda sheep. Ouda and Balami sheep were therefore genetically related.

There is need for complete characterization, genotyping and finding the allele frequencies of casein gene of indigenous sheep breeds, this will offer the possibility to get a complete picture about milk protein gene and to consider milk protein variation in specific breeding programmed in improving consumer preference. In conclusion, casein CSN1S1 was isolated in Balami sheep within 154 bp of chromosome 6, intron 16 and exon 17. It was characterized, shown polymorphism, genetic variation within and between breeds. These sequence obtained from all breeds will be deposited on NCBI data base for further research. This will assists in conserving the genes of the native animals for breeding purposes.

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Review Article

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Plants as Potential Repellent Against *Oryzaephilus* Species

Nurul Huda A.[1*], Nor Hafizah Zakaria[2], Maizatul Akma Ibrahim[1]

ABSTRACT
Stored food pests are a perennial problem in storage facilities and retail stores where they infest and contaminate on a variety of products including grain products, dried fruits, nuts, seeds, dried meats, and in fact, almost all plant products that were used as human foods. The utilization of synthetic pesticides as the main strategy to control food pests has long attracted major concern due to the residue problems and adverse effects to consumers. In view of the above, there is an increasing extensive search for plant species that are showing insecticidal and repellent properties to eradicate these pests that feed on the stored products. These harmful pests include *Oryzaephilus surinamensis* Linnaeus which is the subject of this review. This review describes the biology of *O. surinamensis* L. and summarizes on the current state of the alternative methods using plant as a repellent to control this species and other stored product pests within the same niche.

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Introduction

The insect infestation affects the food manufacturing and other industries greatly. Recently, many controls have been developed in order to avoid this pest attack from happening. The methods used include chemical, biological, and cultural control (i.e Integrated Pest Management). However, in spite of the use of all available controls, the pest infestation still occurred especially in household. The main insect pests that can cause a huge threat to stored-product are khapra beetle (*Trogoderma granarium* Everts), rice weevil (*Sitophilus oryzae* Linnaeus), red flour beetle (*Tribolium castaneum* Herbst), drug store beetle (*Stegobium paniceum* Linnaeus), cigarette beetle (*Lasioderma serricorne* Fabricius), lesser grain borer (*Rhyzopertha dominaca* Fabricius), long headed flour beetle (*Latheticus oryzae* Waterhouse), saw-toothed grain beetle (*Oryzaephilus surinamensis* Linnaeus), rice moth (*Corcyra cephalonica* Stainton), cowpea weevil (*Callosobruchus maculatus* Fabricius) and angoumois grain

[1] Department of Plant Science, Kulliyyah of Science, International Islamic University Malaysia (IIUM), Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia.
[2] Department of Biotechnology, Kulliyyah of Science, International Islamic University Malaysia (IIUM), Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia.
*Corresponding Author: Nurul Huda A., e-mail: anhuda@iium.edu.my

moth (*Sitotroga cerealella* Olivier) [1]. Some pests prefer storage containers, grain silos and warehouses as their home. These pests will damage the raw materials and stored-product through their feces, web and cast skin.

Among the insects mentioned, *Oryzaephilus* (Coleoptera: Silvanidae) species is a very common pest that feed of a variety of foodstuffs [2]. They are secondary pests in stored grain due to their habit to eat only some part from whole grain. However, this species can cause a mechanical damage to the grains in storage facilities, where this pest population will gradually increase and cause a huge infestation problems [3]. *Oryzaephilus mercator* Fauvel (merchant grain beetle), *Oryzaephilus acuminatus* Halstead (grain beetle) and the sawtoothed grain beetle, *Oryzaephilus surinamensis* Linnaeus are the most common species from this genus. Morphologically, these species were similar with each other. They might be differed based on the width of the temple. Both *O. mercator* Fauvel and *O. acuminatus* Halstead have relatively big eyes and short temples, while *O. surinamensis* L. posses small eyes and long temples [4, 5]. The temple of sawtoothed grain beetle is at least one-half length of the eyes. While, the temple of mercant grain beetle is only one-third length of the eye.

These species are abundant and widespread. They can be found in various foodstuffs and habitats, particularly in fodder storages, retail stores, mills, and restaurants [6, 7]. However, because they live in the bottom layers of infested food products, they are difficult to be detected. Frequently, the merchant grain beetle can be detected in higher oil contents products such as nuts and olive. Other than that, this species also feed on seed-borne fungi [7]. Meanwhile, the sawtoothed grain beetle is a polyphagous species that infested mostly on the foods that have high content of carbohydrates such as oats, barley, sugar, dried meat, cereal, rice, dried fruit, seeds, and less often on processed foods such as confectioneries and bread [8].

The needs to control the infestation of *Oryzaephilus* sp. has become a major issue because up to 6% of the raw materials can be damaged due to this problem especially during harvest, transport and storage [9]. While, Matthews [10] added that insect damage might cause for 10-40 % of loss worldwide. In the Kenyan highlands, total loss due to pest in maize were estimated at 57% with insect pest being more important than diseases [11]. According to Pinto [12], the damage caused by overall insect activity range from 0.2 % to 30 % of the grain production particularly due to the poor storage

condition in Brazil. In terms of monetary value, Indian agriculture industry suffered a loss of US$ 36 billion annually due to insects and pests damage [13]. Meanwhile in Malaysia, post-harvest loss of rice at commercial level caused by these insects was reported around 20-44% [14]. Based on a study conducted by Syarifah Zulaikha [15], *O. surinamensis* L. showed the highest abundance in three rice storage facility (Tenggara, Jasa, Target Lane) in Klang with total 47403 individuals recorded during the study period. These data have shown that insect pest of stored food products prevalent in many countries and caused variable losses. Therefore, it is important to have safe methods (i.e using semiochemicals derived from plants) of preservations and control for this pest to avoid major economic loss.

Description and life cycle of Oryzaephilus surinamensis L.

Among all stored product pests, the sawtoothed grain beetle *O. surinamensis* L. is one of the most widespread species, and their infestation can originate at the manufacturing, storage or retail levels. The distribution of the beetle is influenced by many factors such as processing techniques, food availability, environmental conditions and interaction between pest species. Foods that may be infested include cereals, flours, pastas, dried fruits, nuts, dries meats, candies, and other similar packaged goods [16].

As accurate identification is key to a successful pest management, here in this part we provided brief description about morphology and life cycle of target pest; sawtoothed grain beetle *O. surinamensis* L. Adults beetle usually are less than 3 mm long, with long, narrow and flattened chocolate brown bodies. Figure 1 showed the morphometric characterizations of *O. surinamensis* L. reared in oat grout under laboratory conditions (27℃ and 64% RH) at Kulliyyah of Science, International Islamic University Malaysia. Females and males are almost similar but males are slightly more elongated in shape than females. According to Barnes [17], in their culture, males range in length from 3.4 to 3.7 mm and females from 3.3 to 3.5 mm. Other than that, the head of the males is broader, while in females, the head slightly narrower. Males also have the posterior margin of the hind trochanter and the upper margin of the hind femur with a spine-like projection. Wings are well developed in both sexes. Along each side of the thorax are six distinctive tooth-like projections [18]. Eggs are elongated, capsuled-shape, about 0.4 mm long, and deposited singly or in small clusters. The larvae are elongated yellowish-white with a brown head has numerous setae (hairs) and three pairs of legs.

They are about 0.8 mm when newly hatched and 3-4 mm when fully developed. The mature larvae are quite active. Adults are relatively long-lived. They can survive for 19 weeks. Adults are also active and they can climb most of vertical surfaces. They do not penetrate packaging materials well but they are fast at gaining access through small holes or gaps in packaging seals. Due to their flattish form, they can pass through holes as small as 0.7 mm in diameter [8].

Oviposition begins shortly after adult emergence (30-35°C and 56–74% relative humidity). Average number of eggs per female is about 280 with maximum numbers of 432. The eggs hatch within 3-8 days [19]. Depending on temperature, the life cycle ranges from 20-80 days. While, depending on the environmental conditions, the adults usually live around 6 to 10 months. Humidity greatly influenced oviposition and development with both decreasing as humidity decreases below 50%. Above 96% relative humidity, the growth for this species is not possible [20]. However, Mallis [21] previously give an opposing view by stated that low humidity only has little effect on the egg period and did not affect the length of the developmental stages.

Fig 1 *Oyzaephilus surinamensis* L. morphometric characterization; a) Length of antenna, b) Head width, c) Length of thorax, d) Body length, e) Body width at the widest point, f) Length of hind femur.

Control of Oryzaephilus species

The control of pests includes the management and regulation of pest which effectively resist further growth of insects and damage done on products. The chemical control is the most popular and effective method. Several publications have shown the efficacy of pesticides such as pirimiphos methyl, phosphine, deltamethrin and chlorpyriphos to control the pest in stored grains i.e Rejesus [22], Arthur [23] and Sgarbiero [24]. The findings showed that these chemical control agents produced effective effects. Phosphine (PH_3) was first introduced in the late 1700s and has been utilized as a grain fumigant since the 1930s [25]. It is by far the most common pest insect control in stored grain. Phosphine has high toxicity to aerobic organisms, but harmless to anaerobic or metabolically dormant organisms. Thus, it can be utilized to kill insect pests in grain, without affecting grain viability. However, in other report, due to its high toxicity, fumigation with phosphine is now seldom used [26]. According to Nath [25], the three hydrides (NH_3 and AsH_3) can disturb of the sympathetic nervous system, suppressed energy metabolism and toxic changes to the redox state of the cell.

The chemical method is probably the commonest and main means in pest management especially in foods and crops such as fruit, vegetables and grains but it has some disadvantages [27]. First, the materials used are usually poisonous to human beings and leave residues on food; and secondly, it caused a development of high degrees of resistance in many insect pests due to continuous use of commercially available synthetic pesticides, thus making controls more difficult. These chemicals also resulted in resurgence and outbreak of new pests which previously regarded as non-insect pests (due to their low population in nature). Furthermore, pesticide contamination is disrupting the ecosystem sustainability by severely affecting beneficial insects, microbes, plants, fishes, birds and other animals [28]. Although effective, they can have a disastrous effect on a habitat's food web. As our knowledge of the side effects of modern broad-spectrum pesticides and fumigants has increased, scientists have started to find new ways to control pest infestation. It appears to be advisable, therefore, to implement the other means rather than the chemical method.

Apart from that, biological control also can be used in order to save the product from *O. surinamensis* L. attack. There is one potential natural enemies of *O. surinamensis* L. which is the parasitoid wasp, *Cephalonomia tarsalis* Ashmead (Hymenoptera:

Bethylidae) reported from Iran [29]. Entomogenous fungus *Beauveria bassiana* (Bals.) Vuill also was identified to have potential to reduce breeding success of *O. surinamensis* L. [30]. However, biological control is not commonly use and mostly still in experimental stage.

Other control methods including physical control using inert dust such as diatomaceous earth [31, 32], ionizing irradiation [33], light and sound [34], thermal regulation and ozonation [35] and semiochemical control or chemical derived from plants to control insect pests by behavioural manipulation (i.e insecticides, repellents or antifeedants) [36, 37]. This review focuses on potential plant as semiochemical of *Oryzaephilus* species and other control methods are not discussed in details.

Plant as semiochemical control; biopesticides, natural repellents and antifeedants

Biopesticides or 'biological pesticides' are naturally occuring substances that are extracted or derived from such natural materials as plants, animals, bacteria, and certain minerals. Biopesticides have gained a great attention in recent years because they offer a safe, friendly, and integrated environmental development. At present, biopesticides are successfully implemented in food industry to manage (either to prevent or delay) the development of pests in food products. Another benefit of biopesticides is they appear to be safe during accidental contacts with higher animals such as human and other mammals [38]. Plant-derived insecticides (targeted only on insects) can replace synthetic chemical insecticides as alternatives to control pest in food products. This is because plants and herbs usually posses only little health risk to human. Moreover, the production and processing costs are low. Biopesticides are in the form of powder, essential oil or solvent extract such as pyrethrum extracted from *Tanacetum cinerariifolium* (Trevir.) Sch. Bip. (Asteraceae) or neem from *Azadirachta indica* A. Juss (Meliaceae) are popular in the market [39, 40].

Meanwhile, repellents are the substances that cause insects to direct their movement away from the food source [41]. Insects will detect the repellent from a short distances and causing them to move away [42]. Plant extracts are safe, non-poisonous, less toxic (not true in all cases) and biodegradable. Traditionally, plant-based repellents have been used as a personal protection method against pests [43, 44]. Through ethnobotanical studies, knowledge on traditional repellent plants obtained is beneficial for the development of new natural products [45]. Plant-based repellent can protect against the

pest with minimal impact on the ecosystem, as they keep the insect pest away from the food products by stimulating olfactory or other receptors. Plant derived repellents are safe in pest control and able to minimize pesticide residue. The safety of the people, food, and environment are also guaranted [43].

As antifeedant, allelochemicals from plants target on specific sensory cells (i.e antifeedant receptors) in the pest or insect. The neurons associated with these antifeedant receptors either prevent insect feeding (feeding deterrent effect) or cause cessation or slowing of further feeding (feeding suppressant effect) [46]. In other words, the chemicals interupt insect feeding by rendering the treated materials unattractive or unpalatable. Another mode of action of some antifeedants is by blocking the function of pest feeding-stimulant receptors, or an ability to bind directly to its normal feeding cues, such as sugars and amino acids therefore disrupting the sensitivity of sugar-sensing cells in insect pest and thus causing the insects to incorrectly assess nutritional adequacy of treated host [37]. For example, study of antifeedant property in *Vernonia oocephala* Baker (Asteraceae) has highlighted the potential of this plant in reducing feeding activity of stored product pests particularly *Tribolium castaneum* Herbst as this could be due to saponins, glycosides, and alkaloids found in the plant extract [47].

Diversity in plant forms as semiochemical against stored product pests

Plant repellent can be formed from plant extracts, raw/powders and essential oil [48] which are extracted from many parts of the plant such as roots, leaves, seed and bark. İt is reported that these plant parts also able to reduce oviposition rate and suppress adult emergence of stored product insects, and also reduce seed damage rates [49]. Several studies also have shown that plant families namely Annonaceae, Asteraceae, Canellaceae, Lamiaceae Meliaceae, and Rutaceae are the most promising natural repellents [50, 51, 52].

Essential oils repel insect with their effect lasting from several minutes to several hours. Essential oils are formed by plants as secondary metabolites [53]. They are volatile and has a strong odour (concentrated extract that retain the natural smell and flavour of the source). Essential oils that have insect repellent activity usually contain allelochemicals like the monoterpenes such as cineole, pinene, eugenol, limonene, citronellol, terpinolene, citronellal, camphor and thymol [54]. The most common essential oil is extracted from Neem. Neem plant *Azadirachta indica* A. Juss (Meliaceae) is a bitter

tonic that has parasiticidal, insecticidal, spermicidal properties and hence destroys a wide range of organism. Ahmed [55] and Talukder [56] reported that 1 to 2% of the neem oil shown to be effective against stored grain insect pests like *Oryzaephilus* sp., *Sitophilus oryzae* Linnaeus, *Tribolium castaneum* Herbst, *Rhyzopertha dominica* Fabricius, and *Callosobruchus chinensis* Linnaeus. According to these researchers, the neem oil bind to the grains and protect against storage pests for 180 to 330 days.

Other than Neem, the essential oil of *Artemisia annua* Linnaeus (sweet wormwood) from Family Asteraceae also has been utilized as a repellent against *Tribolium castaneum* Herbst and *Callosobruchus maculatus* Fabricius [57]. In northern Cameroon, the essential oils of *Xylopia aethiopica* (Dunal) A. Rich. (Annonaceae), *Vepris heterophylla* (Engl.) Letouzey (Rutaceae) and *Lippia rugosa* A. Chev (Verbenaceae) were also applied to protect from the infestatation of stored grain insect pests [58]. In other study by Zia [59], the essential oil from citrus peels was used as grain protectants against *Callosobruchus chinensis* L., *Trogoderma granarium* Everts and *Tribolium castaneum* Herbst. They observed that depending on concentrations and exposure durations, the essential oil showed variable toxicity to insects. Due to similarity in behavior and niche, similar results also can be expected occurred on *O. surinamensis* L.

Meanwhile, Al-Jabr [19] in his study evaluated the application of several essential oils such as *Cinnamomum camphora* (L.) H. Karst (Lauraceae), *Cymbopogon winterianus* Jowitt (Poaceae), *Matricaria chamomilla* Linnaeus (Asteraceae), *Mentha viridis* Linnaeus (Lamiaceae) , *Prunus amygdalus* var. *amara* (DC.) Buchheim (Rosaceae), *Rosmarinus afficinalis* Linnaeus (Lamiaceae) and *Simmondsia chinensis* (Link) CK. Schneid (Simmondsiaceae). The toxicity and repellent effectiveness against adults of *O. surinamensis* L. and *Tribolium castaneum* Herbst were tested using these plants extracts. Their results showed that essential oil of *Mentha viridis* Linnaeus, *Matricaria chamomilla* Linnaeus and *Cinnamomum camphora* (L.) H. Karst. showed a high mortality of *O. Surinamensis*. Similar results regarding repellant activity of *Cinnamomum camphora* (L.) H. Karst. on other insect pests also have been reported by many authors such as Liu [60], Cansian [61] and Guo [62]. Other plant oil that has been found effective against stored product pest is coconut oil [63].

Rajashekar [52] on the other hand, highlighted that dried leaves (raw form) of *Azadirachta indica* A. Juss (Meliaceae) was effective against insects when mixed with

stored grains. In term of availability, raw form (without extraction) is the best form to be studied their potential as repellent against stored product pests as it readily available for household use compared to essential oil and solvent extract. A study by Klys [64] has investigated effectiveness of the plant powders of peppermint *Mentha piperita* Linnaeus (Lamiaceae), wormwood *Arthemisia absinthium* Linnaeus (Asteraceae), common sage *Salvia officinalis* Linnaeus (Lamiaceae), allspice *Pimenta dioica* (L.) Merrill (Myrtaceae) and common garlic *Allium sativum* Linnaeus (Amaryllidaceae) used in different concentrations on the mortality rates of the *O. surinamensis* L. The result showed that at the concentration of 1.23%, all spice seeds powder demonstrated the highest mortality among *O. surinamensis* L. Meanwhile, the powder of sage, peppermint and wormwood caused the highest statistically significant mortality of *O. surinamensis* L when the concentrations of 3.61 and 5.88% were used.

Rajashekar [52] also reported that a significant finding was shown when the root powder of *Decalepis hamiltonii* Wight & Arn. (Apocynaceae) was tested against various stored grain insect pests. Other than that, extraction from powder (using distilled water) of ginger *Zingiber officinale* Roscoe (Zingiberaceae) and caraway *Carum carvi* Linnaeus (Apiaceae) and cardamom *Elettaria cardamomum* Linnaeus (Zingiberaceae) by Amiri [65] demonstrated that high repellency was detected in ginger and caraway on larvae and adult of *O. surinamensis* L. and *O. mercator* Fauvel than cardamom. In their study, a high repellent effect was detected for all plant extracts with the increased concentrations and time of exposure. While, in an experiment set up by Devi [66] the powders of 17 spices such as mace, pepper, nutmeg, cloves, cinnamon, star anise, fennel, ajowan, cumin, caraway, turmeric, ginger, bay leaves, red chilies, cappers, coriander, and fenugreek were evaluated for their insecticidal, antifeedant and antiovipositional potential against *Sitophilus oryzae* Linnaeus infesting wheat. The results showed that these spices powder showed an effective protection to wheat up to 9 months without affecting seed germination thereby showing promise as grain protectants. On the other hand, Tiwari [67] reported that the powders of *Rauvolfia serpentina* (L.) Benth. ex Kurz., *Acorus calamus* Linnaeus (Apocynaceae) and *Mesua ferrea* Linnaeus (Calophyllaceae) showed a positive finding as grain protectants against *Rhyzoperta dominica* Fabricius.

Meanwhile, solvent extracts of many plants have shown varying levels of insect-repellent properties. Commonly used solvents are ethyl acetate, diethyl ether and dichloromethane. The repellent effects of solvent extracts of indigenous plants were tested against *O. surinamensis* L. by Shah [68]. According to the authors, *Typhonium trilobatum* (L.) Schott (Araceae), *Cleome viscosa* Linnaeus (Capparidaceae), *Cassia occidentalis* Linnaeus (Fabaceae), *Pongamia pinnata* (L.) Pierre (Fabaceae), *Mensua ferrea* Linnaeus (Calophyllaceae) and *Trewia nudiflora* Linnaeus (Euphorbiaceae) showed the highest repellency rate at 10.0% dose level. Manzoor [69] reported that *O. surinamensis* L. were repeled by the ethanolic extract of five plant leaves; bakain *Melia azedarach* Linnaeus (Meliaceae), datura *Datura stramonium* Linnaeus (Solanaceae), lemongrass *Cymbopogon citratus* Stapf. (Poaceae), mint *Mentha longifolia* (L.) Huds. (Lamiaceae) and habulas *Myrtus communis* Linnaeus (Myrtaceae). The result demonstrated that lemongrass showed the maximum number of repellent (based on number of alive insects) after 48 hour. In addition, Dwivedi [70] observed that *Cassia occidentalis* Linnaeus (Fabaceae) and other aboriginal plant species showed possible repellent action of against a stored product pest insect under laboratory conditions. From their observation, the acetone extracts showed good repellent effect towards the tested insect.

Other than studies that have been reviewed above, many other research have been done on plants in order to find method which does not burden producers, retailers and consumers financially, safer for environment and quite effective to control stored product pests. Table 1 listed some of the studies on potential plants species as repellent against *O. surinamensis* together with other stored product insects. From an economical point of view, synthetic chemicals are still more popular as repellents than plant-based. However, for a safer repellents for humans and the environment, these natural products should be considered as an alternative to the synthetic chemicals.

Table 1 List of plant species reported to show insecticidal or repellent activity on *O. surinamensis* and other stored product insects from the same study

No.	Plant species	Family	Plant parts	Stored product pests	References
1.	*Agastache foeniculum* (Pursh) Kuntze	Lamiaceae	Aerial parts	*Oryzaephilus surinamensis* L. *Lasioderma*	[71]

				serricorne Fabricius	
2.	*Ageratum conyzoides* Linnaeus	Asteraceae	Leaves	*Oryzaephilus surinamensis* L. *Rhyzopertha dominica* Fabricius *Sitophilus oryzae* L.	[72]
3.	*Ailantus altissima* (Mill.) Swingle	Simaroubaceae	Bark	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst *Sitophilus oryzae* L. *Liposcelis paeta* Pearman	[73]
4.	*Allium cepa* Linnaeus	Amaryllidaceae	Bulbs* Bulbs	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst *Oryzaephilus surinamensis* L.	[74] [75]
5.	*Allium sativum* Linnaeus	Amaryllidaceae	Bulbs	*Oryzaephilus surinamensis* L.	[64], [75]
6.	*Anethum graveolens* Linnaeus	Apiaceae	Seeds	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst	[76]
7.	*Argemone ochroleuca* Sweet	Papaveraceae	Leaves	*Oryzaephilus surinamensis* L.	[77]
8.	*Artemisia absinthium* Linnaeus	Asteraceae	Whole plant Leaves	*Oryzaephilus surinamensis* L. *Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst	[64] [78]
9.	*Artemisia argyi* Levl. et	Asteraceae	Whole plant	*Oryzaephilus surinamensis* L.	[79]

	Vant.				
10.	*Artemisia herba-alba* Asso.	Asteraceae	Leaves	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst	[78]
11.	*Azadirachta indica* A. Juss	Meliaceae	Leaves Leaves, Seed kernels	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst *Oryzaephilus surinamensis* L. *Acanthoscelides obtectus* Say *Sitophilus oryzae* L. *Cryptolestes ferrugineus* Stephens	[80] [81]
12.	*Calotropis procera* (Ait.) Ait.	Apocynaceae	Leaves	*Oryzaephilus surinamensis* L.	[82]
13.	*Cananga odorata* Hook. f. et Thomson	Annonaceae	Flowers	*Oryzaephilus surinamensis* L.	[83]
14.	*Capparis spinosa* Linnaeus	Capparaceae	Leaves	*Oryzaephilus surinamensis* L.	[77]
15.	*Caralluma tuberculata* N.E. Brown	Apocynaceae	Leaves	*Oryzaephilus surinamensis* L.	[77]
16.	*Carum carvi* Linnaeus	Apiaceae	Fruits Seeds* Seeds	*Oryzaephilus surinamensis* L. *Oryzaephilus mercator* Fauvel *Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst *Oryzaephilus surinamensis* L.	[65] [74] [75]

17.	*Cassia occidentalis* Linnaeus	Fabaceae	Leaves	*Oryzaephilus surinamensis* L.	[68]
18.	*Chenopodium album* Linnaeus	Amaranthaceae	Leaves	*Oryzaephilus surinamensis* L.	[41]
19.	*Cinnamomum camphora* (L.) H. Karst	Lauraceae	Wood*	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst	[19]
20.	*Citrus aurantium* Linnaeus	Rutaceae	Leaves	*Oryzaephilus surinamensis* L. *Lasioderma serricorne* Fabricius	[84]
21.	*Cleome viscosa* Linnaeus	Cleomaceae	Leaves	*Oryzaephilus surinamensis* L.	[68]
22.	*Cordia verbenacea* A. DC.	Boraginaceae	Leaves	*Oryzaephilus surinamensis* L. *Rhyzopertha dominica* Fabricius *Sitophilus oryzae* L.	[672]
23.	*Crataegus sinaica* Boisser	Rosaceae	Leaves	*Oryzaephilus surinamensis* L. *Carpophilus hemipterus* L.	[85]
24.	*Cymbopogon citratus* Stapf	Poaceae	Leaves Whole plant	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst *Callosobruchus chinensis* L. *Oryzaephilus surinamensis* L. *Sitophilus zeamais* Motschulsky	[69] [86]
25.	*Cymbopogon martini*	Poaceae	Whole plant	*Oryzaephilus surinamensis* L.	[86]

	(Roxb.) W.Watson			*Sitophilus zeamais* Motschulsky	
26.	*Cymbopogon nardus* (L.) Rendle	Poaceae	Whole plant	*Oryzaephilus surinamensis* L. *Sitophilus zeamais* Motschulsky	[86]
27.	*Cymbopogon winterianus* Jowitt	Poaceae	Aerial parts*	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst	[19]
28.	*Cyperus fuscus* Linnaeus	Cyperaceae	Rhizomes*	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst	[74]
29.	*Datura stramonium* Linnaeus	Solanaceae	Leaves	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst *Callosobruchus chinensis* L. *Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst *Oryzaephilus surinamensis* L. *Oryzaephilus surinamensis* L. *Rhyzopertha dominica* Fabricius *Sitophilus oryzae* L.	[69] [80] [82] [72]
30.	*Elettaria cardamomum* Linnaeus	Zingiberaceae	Fruits Seeds	*Oryzaephilus surinamensis* L. *Oryzaephilus mercator* Fauvel *Oryzaephilus surinamensis* L.	[65] [75]

			Fruits + Seeds	*Oryzaephilus surinamensis* L.	[87]
31.	*Eucalyptus dundasii* Maiden	Myrtaceae	Leaves	*Oryzaephilus surinamensis* L *Rhyzopertha dominica* Fabricius	[88]
32.	*Eucalyptus floribunda* F. Muell.	Myrtaceae	Leaves	*Oryzaephilus surinamensis* L. *Rhyzopertha dominica* Fabricius	[89]
33.	*Eucalyptus globulus* Labill.	Myrtaceae	Leaves*	*Oryzaephilus surinamensis* L.	[90]
34.	*Foeniculum valgare* Miller	Umbelliferae	Seeds Fruits + Seeds	*Oryzaephilus surinamensis* L. *Oryzaephilus surinamensis* L.	[75] [87]
35.	*Fragaria ananassa* Duch.	Rosaceae	Fruits	*Oryzaephilus surinamensis* L.	[75]
36.	*Illicium verum* Hook. F.	Schisandraceae	Flowers	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst	[76]
37.	*Lantana camara* Linnaeus	Verbenaceae	Leaves	*Oryzaephilus surinamensis* L.	[41]
38.	*Lavandula augustifolia* Miller	Lamiaceae	Flowers*	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst *Oryzaephilus surinamensis* L.	[74] [83]
39.	*Leonotis nepetifolia* (L.) R. Brown	Lamiaceae	Leaves	*Oryzaephilus surinamensis* L. *Rhyzopertha dominica* Fabricius	[72]

				Sitophilus oryzae L.	
40.	*Lepidoploa aurea* (Mart. ex DC.) H. Robinson	Asteraceae	Leaves	*Oryzaephilus surinamensis* L. *Sitophilus zeamais* Motschulsky *Tribolium castaneum* Herbst	[91]
41.	*Linum usitatissimum* Linnaeus	Linaceae	Seeds*	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst	[74]
42.	*Marrubium vulgare* Linnaeus	Lamiaceae	Leaves	*Oryzaephilus surinamensis* L.	[77]
43.	*Matricaria chamomilla* Linnaeus	Asteraceae	Flower*	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst	[19]
44.	*Maytenus emarginata* (Willd.) Ding Hou	Celastraceae	Leaves	*Oryzaephilus surinamensis* L.	[41]
45.	*Melia azedarach* Linnaeus	Meliaceae	Leaves	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst *Callosobruchus chinensis* L.	[69]
46.	*Memora nodosa* Miers	Bignoniaceae	Flowers	*Oryzaephilus surinamensis* L. *Sitophilus zeamais* Motschulsky *Tribolium castaneum* Herbst	[91]
47.	*Mentha longifolia* (L.) Hudson	Lamiaceae	Leaves	*Oryzaephilus surinamensis* L. *Tribolium*	[69]

				castaneum Herbst *Callosobruchus chinensis* L. *Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst	[80]
48.	*Mentha piperita* Linnaeus	Lamiaceae	Leaves	*Oryzaephilus surinamensis* L. *Oryzaephilus surinamensis* L. *Rhyzopertha dominica* Fabricius *Sitophilus oryzae* L.	[64] [72]
49.	*Mentha viridis* Linnaeus	Lamiaceae	Leaves*	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst	[19]
50.	*Mesua ferrea* Linnaeus	Calophyllaceae	Leaves	*Oryzaephilus surinamensis* L.	[68]
51.	*Mormodica charantia* Linnaeus	Cucurbitaceae	Leaves	*Oryzaephilus surinamensis* L. *Rhyzopertha dominica* Fabricius *Sitophilus oryzae* L.	[72]
52.	*Myrtus communis* Linnaeus	Myrtaceae	Leaves	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst *Callosobruchus chinensis* L.	[69]
53.	*Nigella sativa* Linnaeus	Ranunculaceae	Seeds	*Oryzaephilus surinamensis* L.	[75]
54.	*Ocimum basilicum* Linnaeus	Lamiaceae	Leaves*	*Oryzaephilus surinamensis* L.	[90]

55.	*Ocimum gratissimum* Linnaeus	Lamiaceae	Whole plant	*Oryzaephilus surinamensis* L. *Rhyzopertha dominica* Fabricius *Tribolium castaneum* Herbst *Callosobruchus chinensis* L. *Sitophilus oryzae* L.	[92]
56.	*Ocimum selloi* Benth.	Lamiaceae	Leaves	*Oryzaephilus surinamensis* L. *Rhyzopertha dominica* Fabricius *Sitophilus oryzae* L.	[72]
57.	*Pimenta dioica* (L.) Merrill	Myrtaceae	Seeds	*Oryzaephilus surinamensis* L.	[64]
58.	*Pongamia pinnata* (L.) Pierre	Fabaceae	Leaves	*Oryzaephilus surinamensis* L.	[68]
59.	*Prunus amygdalus* var. *amara* (DC.) Buchheim	Rosaceae	Seed kernels*	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst	[19]
60.	*Prunus laurocerasus* Linnaeus	Rosaceae	Leaves	*Oryzaephilus surinamensis* L. *Carpophilus hemipterus* L.	[85]
61.	*Punica granatum* Linnaeus	Lythraceae	Leaves	*Oryzaephilus surinamensis* L.	[41]
62.	*Pyracantha coccinea* Roemer	Rosaceae	Leaves	*Oryzaephilus surinamensis* L. *Carpophilus hemipterus* L.	[85]
63.	*Rhazya stricta* Decne.	Apocynaceae	Leaves	*Oryzaephilus surinamensis* L.	[77]

64.	*Rosmarinus officinalis* Linnaeus	Lamiaceae	Leaves + young stems*	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst	[19]
65.	*Ruta graveolens* Linnaeus	Rutaceae	Leaves	*Oryzaephilus surinamensis* L. *Rhyzopertha dominica* Fabricius *Sitophilus oryzae* L.	[72]
66.	*Salvia officinalis* Linnaeus	Lamiaceae	Whole plant Leaves + Flowers	*Oryzaephilus surinamensis* L. *Oryzaephilus surinamensis* L.	[64] [75]
67.	*Simmondsia chinensis* (Link) C.K. Schneid	Simmonsiaceae	Seeds*	*Oryzaephilus surinamensis* L. *Tribolium castaneum* Herbst	[19]
68.	*Solenostemma argel* (Del.) Hayne	Apocynaceae	Leaves	*Oryzaephilus surinamensis* L.	[782]
69.	*Sorbus aucuparia* Linnaeus	Rosaceae	Leaves	*Oryzaephilus surinamensis* L. *Carpophilus hemipterus* L.	[85]
70.	*Syzygium aromaticum* (L.) Merr. et L.M. Perry	Myrtaceae	Seeds	*Oryzaephilus surinamensis* L.	[75]
71.	*Thymus vulgaris* Linnaeus	Lamiaceae	Leaves	*Oryzaephilus surinamensis* L.	[75]
72.	*Trewia nudiflora* Linnaeus	Euphorbiaceae	Leaves	*Oryzaephilus surinamensis* L.	[68]
73.	*Triticum aestivum* Linnaeus	Poaceae	Seeds	*Oryzaephilus surinamensis* L.	[75]

74.	*Typhonium trilobatum* (L.) Schott	Araceae	Leaves	*Oryzaephilus surinamensis* L.	[68]
75.	*Vitex negundo* Linnaeus	Lamiaceae	Leaves	*Oryzaephilus surinamensis* L.	[41]
76.	*Zingiber officinale* Roscoe	Zingiberaceae	Fruits Rhizomes	*Oryzaephilus surinamensis* L. *Oryzaephilus mercator* Fauvel *Oryzaephilus surinamensis* L.	[65] [87]

* Commercially available essential oils.

Conclusion

Biological and Chemical studies of protective allelochemicals in plants especially those disrupting pest functional activity is important for future efforts to control the damage in crops due to pest infestation. Due to this fact, many researchers have evaluated the insecticidal properties of plant-based repellents on various species of stored product insect pests. The results clearly showed that the application of plant-based products as alternative to synthetic chemicals is proven to be more effective, sustainable and safe with low toxicity effect on non-target organisms. Some of plant products can work not only on *Oryzaephilus surinamensis* Linnaeus but simultaneously against many other pest species like *Tribolium castaneum* Herbst and *Sitophilus oryzae* Linnaeus. In addition to that, the pest management will be more efficient and impactful if some measures were taken such as early detection, effective monitoring and knowledge of the way of life and habits of pest species.

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Availability of data and material
Please contact the corresponding author for any data request.

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