RESEARCH ARTICLE

SABRAO Journal
of Breeding and Genetics
45 (3) 391-399, 2013

EARLY DETECTION OF SUPERIOR VARIETIES OF SAINFOIN (*Onobrychis sativa*) THROUGH *IN VIVO* AND *IN VITRO* STUDIES

SADEGH MOHAJER[1*], ROSNA MAT TAHA[1], HASHIMAH ELIAS[1], ALOKA KUMARI[2] and MAMALAY RUKK SANNA[1]

[1] Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.
[2] Plant Systematic Research Centre, Department of Botany, Bhagalpur University, 812007 Bhagalpur, India
*Corresponding author's email: mohajer.ae@gmail.com

SUMMARY

In order to detect superior varieties at early growth stage of Sainfoin (*Onobrychis sativa*) varieties, *in vivo* and *in vitro* grown sainfoin seedlings (4 weeks old) from 2 separate experiments were assessed by using complete block design with three replications. The collected data were shoot length, radicle length, seedling dry weight, germination percentage, vigor index, number of tillers and rootlets. These results indicated that *in vitro* growth condition has an advantage over *in vivo* condition since Nitrogen is available to be used directly as NH3. Based on the results obtained, population of Orumieh-1763 had the best result among varieties in both growth conditions and population of Golpaygan-181 proved to be a sensitive variety. This research suggests a new method to select sensitive and good varieties through comparison of *in vivo* and *in vitro* grown plants, even at seedling stage i.e. during early growth development. The relationship between *in vivo* and *in vitro* grown plants as measured by phenotypic correlation was poor for all traits, except for shoot length and number of rootlets. This suggests that response to selection based on *in vivo* data would not be reflected in *in vitro*, so, for all characters, selection should be based on *in vitro* culture for genetic and plant breeding purposes.

Keywords: *in vitro*, *in vivo*, *Onobrychis sativa*, vigor index, variety, germination percentage

Manuscript received: March 21, 2012; Decision on manuscript: April 26, 2013; Manuscript accepted: August 9, 2013

Communicating Editor: Bertrand Collard

INTRODUCTION

Sainfoin (*Onobrychis sativa*) originates from Turkey, Iran and Europe. It was first cultivated in the north of France (Delgado Munoz, 2008) and in the United Kingdom (Koivisto *et al.,* 2001). It is now widespread in warm-temperate Europe (as far as Sweden), Asia, Mediterranean countries and western North America. Sainfoin has a wider optimum temperature range for germination and early seedling growth than most other forage legumes (Smoliak *et al.,* 1972), performs best on deep, well-drained calcareous soils at pH level of 6.0 and warm temperate climates have vigorous seedling growth (Frame *et al.,* 1998). It is tolerant to drought and cold if nitrogen reserves are high enough. It can be irrigated profitably. As a nitrogen-fixing legume, sainfoin is used as green manure and cover-crop. In abandoned areas and hill slopes where erosion may be a problem, sainfoin seedlings may prevent desertification and erosion and maintain soil fertility. Its melliferous flowers attract bees and birds and enhance biodiversity.

Its high tannin content prevents bloating in ruminants, reducing methane and ammonia

production. It is also interesting to include sainfoin in grass pasture as it lowers the nitrogen fertilization requirements of the grass and thus the nitrogen intake and the subsequent urinary nitrogen output of cattle (Waghorn, 2008). Sainfoin hay has a high palatability and a higher tannin level and metabolizable energy than Lotus corniculatus and Cichorium intybus (Scharenberg *et al.,* 2007). It has higher nitrogen digestion efficiency than alfalfa cut at vegetative stage (Aufrère *et al.,* 2008).

Sainfoin silage has a beneficial effect on dry matter intake, dry matter digestibility and cellulose digestibility in sheep (Tatl *et al.,* 2001) as well as duodenally utilisable crude protein and metabolizable energy (Scharenberg *et al.,* 2007). It is also a good source of macro and micro-minerals, except for Cu, Zn and Mg which need to be supplemented. The nitrogen digestibility of sainfoin silage is lower than that of other legume silages (Fraser *et al.,* 2000).

In vitro research is generally referred to as the manipulation of organs, tissues, cells, and biomolecules in a controlled, artificial environment. The characterization and analysis of biomolecules and biological systems in the context of intact organisms is known as *in vivo* research. Lots of *in vivo* researches are done by scientists who usually focus on forage yield, morphology and quality traits of sainfoin (Anon, 1988; Tosun *et al.,* 1988; Turk and Celik, 2006; Mohajer *et al.,* 2011). Although sainfoin is very important as a forage and soil improvement crop, it has received little attention for *in vitro* studies (Sankac, 1999). A high frequency of adventitious shoot regeneration from a range of explants including mature (Ozcan *et al.,* 1996a) and immature embryos (Ozcan *et al.,* 1996b), leaflets, petioles and stems has also been reported for sainfoin (Ozcan *et al.,* 1998). Recently, *in vitro* shoot regeneration from cotyledon node explants of sainfoin using various growth regulators has been reported by Saglam (2010).

Tissue culture is considered as one of the most important topics in biotechnology. One of the advantages of tissue culture is the supply of a suitable environment to maximize the efficiency of the plant growth. The results of growth parameters such as morphology and seed traits in *in vitro* situation can be documented and compared with the *in vivo* conditions. These kinds of studies were hardly done before. Micropropagation offers a large true- to- type number of plants being produced at the same time. The main aim of this experiment was to compare the *in vivo* and *in vitro* growth parameters through statistical analysis. Other important objectives of the present research were to: 1. Identify and suggest the best varieties of *Onobrychis sativa* to be planted for better yield. 2. Study the relationship of the 2 cultivation conditions (*in vivo* and *in vitro*) through correlation analysis.

MATERIALS AND METHODS

The present work was carried out at Science Faculty, University of Malaya. Ten populations of *Onobrychis sativa* germplasms existing at gene bank of natural resources of Iran were selected. sainfoin (*Onobrychis sativa*) genotypes were assessed in two separate experiments under *in vitro* and *in vivo* conditions by using complete block design with three replications (30 seeds per replication).

In vitro culture

The seeds were soaked in distilled water for 20 minutes with addition of 1-2 drops of Tween-20. The Seeds were sterilized by soaking in sodium hypochlorite (chlorox) solution of 70%, 50%, 30% and 10% for 5 min each. After that the seeds were rinsed 3 times with sterile distilled water for 5 min. They were surface sterilized with 70% alcohol in the laminar flow. Finally the seeds were rinsed again with sterile distilled water 3 times.

Stock solutions of MS (Murashige and Skoog, 1962) media used in this study that were prepared by dissolving the required amount of macro and micro nutrients and vitamins in 1 L of distilled water and kept in dark-colored bottles in a refrigerator. The recommended amount of each stock solution was added to distilled water up to 75% of final volume required for the medium preparation. To prepare the Murashige and Skoog medium (MS), 3% (w/v) sucrose and 0.75% (w/v) agar (different examinations showed roots of sainfoin deform in medium with

more than 0.75% agar) without hormone were added to stock solution. The pH of the medium was adjusted to 5.8 using 1 N NaOH or 1N HCl prior to autoclaving.

Autoclaving was carried out at 120 °C and 20 psi for 20 min. The laminar air flow cabinet was sterilized by exposing to ultraviolet radiation for at least 1 h before seeds inoculation in media of sterile screwed-cap jam jars. Each variety was cultured in 3 replications with 30 seeds. Cultures were transferred to a growth room and maintained at 24 ± 2 °C and 16-h photoperiod (and 8-h dark period). Light was supplied using white fluorescent tube at a photosynthetic photon flux density of 40-45 µmol m^{-2} sec^{-1}.

***In vivo* seed germination**

The seeds and containers were not sterilized as in previous method. The samples were germinated on pots filled with soil (plantflora humus) in greenhouse. These samples were also kept in growth room with 24 ± 2 °C, 70% humidity and 16-h photoperiod.

Data Recorded

The following traits were measured under *in vivo* and *in vitro* conditions: Average of radicle length (RL) and shoot length (SL) were measured three weeks after germination from normal seedlings selected at random from each replication and expressed in millimeter and those seedlings used for growth measurement were placed in a paper covered and dried in shade for 8 hrs and then they were kept in an oven maintained at 45 ± 2 °C for 24 hr. The dried seedlings were weighed to determine the seedling dry weight (SDW) and the mean values were expressed in g. Ratio of germinated (PG) plants was calculated after 3 weeks and Mean of the tillers (NT) and rootlets (NR) were measured from each replication after 4 weeks

The vigor index (VI) value was computed as described by Abdul-Baki and Anderson (1970) by multiplying germination of seeds in percentage and total seedling length in millimeter following the formula:

Vigor index = [germination percentage x mean (radicle length + plumule length)]/100

Statistical Analysis

Data of each experiment was subjected to analysis of variance (ANOVA) separately. Similarly, data of two culture conditions were put under combined analysis. For determination of superior populations, mean comparison for each seven traits was made in DMRT Duncan test. Correlation coefficient estimation was made between two growth conditions for grasping out the relationship between the two growth conditions. Also, correlation coefficient of phenotype was calculated among traits for data of two culture conditions. In addition, advanced SAS and Minitab software system was used for analyzing data.

RESULTS

The results of analysis of variance (ANOVA) in 2 cultivation methods of *in vivo* and *in vitro* are shown in Table 1. *In vitro* condition, effect of population for all traits at probability level of 5% and 1% showed significant differences, except for seedling dry weight. Number of rootlets and germination percentage were not significant for *in vivo* condition (Table 1). Results of combined analysis of 2 growth conditions showed that effect of culture condition for trait of Seedling dry weight at probability level of 5% and in traits of shoot length, radicle length, number of tillers and vigor Index at 1% level were significantly different. Interaction of variety and growth condition was significant in the shoot and radicle length, number of tiller and vigor index (Table 2). Varieties for shoot length were at probability level of 1% was significantly different *in vitro* culture and populations of Orumieh-1763 and Gorgan-1601 were long-legged with 51.7 and 45.6 mm, while population of Esfahan-182 and Karaj-962 were short-legged with 8.6 and 10.6 mm, respectively. Population of Orumieh-1763 was the tallest in *in vivo* with 25.1 mm.

Table 1. ANOVA of traits *in vivo* and *in vitro* condition.

in vivo	df	SL	RL	SDW	NT	NR	GP	VI
Block	2	146.5	12.4	0.0003	0.22	1.3	104.3	39.6
Population	9	168.5**	187.7**	0.001**	0.77*	18.1	83.9	330.3**
Error	18	913.4	897.1	0.0001	0.37	10.18	186.8	79.7
%CV		50.4	35.7	13.3	58.1	93.9	16.7	32.2
in vitro								
Block	2	95.6	86.3	0.04	0.3	18.6	19.2	132.6
Population	9	684.4**	165.5**	0.06	1.7**	21.7*	279.8*	1009.4**
Error	18	175.3	52	0.03	0.55	8.5	106.5	200.5
%CV		46.1	29.3	54.7	37.3	61.2	13.1	33.2

SL: Shoot Length RL: Radicle Length SDW: Seedling Dry Weight NT: Number of Tillers NR: Number of Roolets
GP: Germination Percentage VI: Vigour Index. *significant at the 0.05 probability level, ** significant at the 0.01 probability level.

Table 2. Summary of combined analysis and the level of significant mean squares for 10 populations of *Onobrychis sativa* under two different growth conditions.

Source	df	SL	RL	SDW	NT	NR	GP	VI
Condition (a)	1	3182.81**	345.6**	0.716*	14.50**	24.70	140.8	3360.8**
Error 1	2	677.71*	111.8	0.021	0.45	27.05	43.4	898.08*
Variety (b)	9	620.66**	211.9**	0.035*	1.52**	33.62**	164.0	877.4**
a * b	9	231.5*	141.3**	0.029	1.16*	5.86	199.8	462.3**
Block	2	347.3	57.3	0.024	0.01	38.80*	80.3	502.6*
Error 2	36	112.9	50.92	0.016	0.46	9.71	146.5	140.19
% CV		39.61	32.2	17.230	44.02	34.27	15.23	23.69

SL: Shoot Length RL: Radicle Length SDW: Seedling Dry Weight NT: Number of Tillers NR: Number of Roolets
GP: Germination Percentage VI: Vigor Index. *significant at the 0.05 probability level, ** significant at the 0.01 probability level.

In comparison of averages between 2 culture conditions, shoot length *in vitro* condition with 28.7 mm was more than *in vivo* condition with 14.1 mm (Table 3). Population of Orumieh-1763 both *in vivo* and *in vitro* had the longest root length with 29.2 and 38.8, respectively. Seedling dry weight was significant only in the *in vivo* growth plants and population of Hamedan-281 had the highest dry weight. *In vitro* culture with 0.331 g was superior to *in vivo* seedlings with 0.113 g. Number of tillers was more *in vitro* compared with *in vivo* condition (Table 4). Plants which have well developed root systems are more efficient in water use and nutrients of soil and resistance to tension (Harrise *et al.,* 2000). There was no significant difference between *in vivo* and *in vitro* culture for number of rootlets but roots were more developed in *in vitro* culture. Population of Orumieh-1763 from *in vitro* and Bijar-624 from *in vivo* growth condition had the highest number of rootlets. Accelerating to germination process is prerequisite of an efficient vegetation cover and high yield (Harris, 1996).

Table 3. Means comparison of traits in 10 populations of *Onobrychis sativa* under *in vivo* and *in vitro* culture.

Trait	Shoot length		Radicle length		Seedling dry weight		Number of tillers	
Population	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*
Shahrkord-18	17.6 ab	19.6 cde	13.6 c	28.2 abc	0.097 cd	0.342 ab	1.64 a	2.34 abc
Golpaygan-181	11.2 bc	41.4 ab	17.4 bc	35.7 a	0.115 bc	0.361 ab	0.82 b	2.43 abc
Esahan-182	7.51 c	8.6 e	16.1 bc	14.6 c	0.111 bcd	0.381 ab	0.57 bc	1.02 c
Hamedan-281	19.4 ab	38.3 abc	15.6 bc	22.3 bc	0.152 a	0.437 ab	1.08 ab	3.34 abc
Bijar-624	5.6bc	39.2 abc	12.4 c	18.4 c	0.107 bcd	0.411 ab	1.38 a	2.33 abc
Kashan-962	3.1 c	10.6 de	15.7 c	28.7 abc	0.117 bc	0.282 b	0.98 ab	1.68 bc
Gorgan-1586	7.4 c	21.3 b-e	23.3 ab	16.1 c	0.112 bcd	0.184 b	10.6 ab	1.05 c
Gorgan-1601	17.2 ab	45.6 ab	18.7 bc	22.5 bc	0.084 d	0.168 b	0.06 c	2.41 abc
Orumieh-1763	25.1 a	51.7 a	29.2 a	38.8 a	0.120 bc	0.643 ab	1.39 a	2.69 ab
Karaj-3001	19.2 ab	35.3 a-d	16.3 bc	33.1 ab	0.101 cd	0.268 b	1.69 a	2.02 abc
Mean	14.1 B	28.7 A	19.7 B	24.5 A	0.113 B	0.331 A	1.05 B	2.03 A
Correlation *in vitro* and *in vivo*	0.57*		0.20		0.35		0.21	

The means of the populations with same small letters were not significantly different as per Duncan's multi-range test at $P < 0.05$.
The total means of the populations under *in vivo* and *in vitro* condition with same capital letters were not significantly different.
**, * : correlation coefficients between conditions were significant at 1% and 5% probability level.

Table 4. Means comparison of traits in 10 populations of *Onobrychis sativa* under *in vivo* and *in vitro* culture.

Trait	Number of Rootlets		Germination Percentage		Vigour Index	
Population	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*
Shahrkord-18	3.32 ab	6.67 ab	85.7 a	85.7 ab	26.8 bc	40.8 bcd
Golpaygan-181	1.66 ab	4.02 ab	80.9 a	88.7 a	24.3 c	66.8 a
Esahan-182	0.24 c	1.26 b	85.6 a	71.1 abc	21.3 c	16.6 d
Hamedan-281	3.71 ab	9.12 a	85.5 a	80.9 abc	29.9 abc	48.8 bc
Bijar-624	6.29 ab	6.72 ab	71.4 a	90.4 a	13.0 c	51.5 abc
Kashan-962	5.32 ab	4.12 ab	78.9 a	76.1 abc	14.6 c	28.9 cd
Gorgan-1586	1.68 ab	3.51 ab	74.1 a	69.3 bc	22.9 c	24.9 cd
Gorgan-1601	0.63 bc	2.31 b	85.7 a	90.4 a	29.6 abc	61.8 ab
Orumieh-1763	7.04 ab	8.02 a	80.9 a	85.8 ab	42.2 a	76.8 a
Karaj-3001	5.28 ab	4.52 ab	85.7 a	62.5 c	30.2 abc	46.9 bc
Mean	3.52 A	4.76 A	81.4 A	78.3 A	27.6 B	42.6 A
Correlation *in vitro* and *in vivo*	0.71**		-0.11		0.36	

The means of the populations with same small letters were not significantly different as per Duncan's multi-range test at $P < 0.05$.
The total means of the populations under *in vivo* and *in vitro* condition with same capital letters were not significantly different.
**, * : correlation coefficients between conditions were significant at 1% and 5% probability level.

In comparing 2 different conditions, the average of 2 conditions for germination percentage was not significantly different. However, the average germination *in vivo* with 81.4% was slightly higher than *in vitro*. Biology and vigor of seeds have much effect on crop yield as well as there is positive relationship between vigor index and forage yield (Harris *et al.,* 2000). Significant difference was observed among populations for vigor index *in vivo* and *in vitro*. Variety of Orumieh-1763 with 42.4 and 76.8 had premier potency *in vivo* and *in vitro* condition. Most varieties when cultured *in vitro* were more stable than *in vivo* and this shows that they have more yield, resistance and quality (Table 4).

The analysis results of dual correlation of traits are shown in Table 5. In line with this, correlation coefficient between vigour index with radical length and shoot length exhibited positive and significant differences at two growth conditions. Relationship between vigour index with number of tillers and germination percentage were positive in *in vitro* condition ($P < 0.01$). Shoot length had positive and significant relation with number of tillers, number of rootlets and germination percentage in *in vitro* culture. This trait also had positive and significant relation with radical length and germination percentage in *in vivo* growth. Seedling dry weight and number of tillers had positive and significant at level of 0.5% *in vitro* culture. Similar to these results were reported by Turk and Celik (2006) in *Onobrychis sativa*, Jafari and Goudarzi (2006) on *Medicago sativa.* This shows that variety with more tillers will have higher forage yield (Table 5). Mean comparison among varieties showed that population of Orumieh-1763 had the best performance in both *in vivo* and *in vitro* growth. This proves that population of Orumieh-1763 can be used widely in both different conditions. Population of Hamedan-281 also can be recommended as a good variety based on both *in vivo* and *in vitro* results (Table 6).

Table 5. Correlation analysis among traits in 10 populations of *Onobrychis sativa* under *in vivo* and *in vitro*.

Traits	Culture	SL	RL	SDW	NT	NR	GP
RL	*in vivo*	0.45*					
	in vitro	0.39					
SDW	*in vivo*	0.15	0.32				
	in vitro	0.42	0.41				
NT	*in vivo*	0.21	-0.08	0.12			
	in vitro	0.73**	0.42	0.53*			
NR	*in vivo*	0.13	-0.14	0.14	0.69**		
	in vitro	0.55*	0.42	0.71*	0.83**		
GP	*in vivo*	0.55*	-0.09	-0.04	-0.17	-0.34	
	in vitro	0.52*	0.02	0.36	0.61*	0.44	
VI	*in vivo*	0.87**	0.81**	0.27	0.05	-0.07	0.39
	in vitro	0.94**	0.58**	0.51*	0.78**	0.59*	0.62**

SL: Shoot Length RL: Radicle Length SDW: Seedling Dry Weight NT: Number of Tillers NR: Number of Roolets GP: Germination Percentage VI: Vigour Index. *significant at the 0.05 probability level, ** significant at the 0.01 probability level.

Table 6. Means comparison of traits in 10 populations of *Onobrychis sativa* under combined analysis.

Population	SL(mm)		RL(mm)		SDW(g)		NT		NR		GP(%)		VI	
Shahrkord-18	18.7	bcd	20.8	c	0.220	ab	2.02	ab	5.20	abc	85.7	a	33.9	bcd
Golpaygan-181	18.2	bcd	30.7	ab	0.165	b	1.16	bcd	1.67	cd	76.2	a	37.4	bc
Esahan-182	8.1	d	15.3	c	0.246	ab	0.74	d	0.67	d	78.6	a	19.0	d
Hamedan-281	28.8	ab	19.0	c	0.295	ab	2.26	a	6.33	ab	83.3	a	39.4	bc
Bijar-624	22.3	bc	15.5	c	0.259	ab	1.83	abc	6.40	ab	80.9	a	32.3	bcd
Kashan-962	6.8	d	21.8	c	0.200	b	1.31	bcd	4.67	a-d	77.5	a	21.8	d
Gorgan-1586	14.4	cd	19.8	c	0.149	b	1.03	cd	2.58	bcd	71.8	a	24.0	cd
Gorgan-1601	31.3	ab	20.3	c	0.127	b	1.24	bcd	1.58	cd	88.1	a	45.7	ab
Orumieh-1763	38.3	a	33.5	a	0.382	a	2.03	ab	7.50	a	83.3	a	59.6	a
Karaj-3001	27.2	abc	24.5	bc	0.184	b	1.84	abc	4.92	abc	74.1	a	38.4	bc

The means of the populations with same small letters were not significantly different as per Duncan's multi-range test at $P < 0.05$.
The total means of the populations under *in vivo* and *in vitro* condition with same capital letters were not significantly different.
**, * : correlation coefficients between conditions were significant at 1% and 5% probability level.

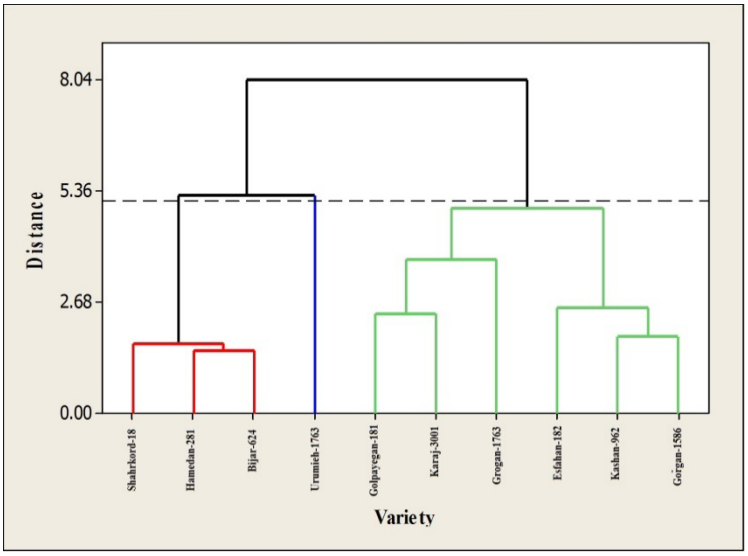

Figure 1. Dendrogram with Ward Linkage and Euclidean Distance in 10 varieties of *O. sativa* under combined analysis.

DISCUSSION

The majority of varieties have same results in both culture conditions but the interesting result was about population of Golpaygan-181. This variety was one of the best populations under *in vitro* culture, however did not have good result in *in vivo* growth condition. Environment, genetic and their interactions have effect on indication of traits. This shows despite Golpaygan-181has a good genome structure for some traits but it is sensitive to environment stresses. So, Golpaygan-181 was considered as a sensitive population. This variety should be cultured in an environment without tension or used as a resource for genetic and breeding studies. Hence, the method applied in the present work may be applied to select some superior and good or sensitive plant varieties in shorter time than some other methods of plant breeding All 7 traits of 10 populations were used and grouped into 3 different categories with dendrogram slice in 5.8 of Euclidean distance in cluster analysis of combination (Figure 1). Cluster analysis also showed that population of Orumieh-1763 had a separate group. In mean comparison of combined analysis, two populations of Esfahan-182 and Gorgan-1586 did not have satisfactory results for 7 traits among 10 varieties. In the most traits, *in vitro* culture was so much better than *in vivo* seedlings especially for vigour index, shoot length and seedling dry weight. In the macronutrients solution of MS media, there is a compound which can prepare NH_3 directly for plant. Ammonium nitrate (NH_4NO_3) separate to NH_4^+ and NO_3^- in water. The ammonium ion is itself a weak acid and hydrolyzes to NH3 and H+.

In this regard, Nakhjavan *et al.,* (2011) also confirm that water soluble carbohydrates (WSC) have positive effect on growing speed of *Onobrychis sativa.* In the present study, although some relations were detected negative among traits in *in vivo* growth condition, but all correlations were observed positive in *in vitro* growth condition which confirms direct relations in *in vitro* traits studied.

ACKNOWLEDGEMENTS

The authors would like to thank the University of Malaya, Malaysia for the facilities and financial support provided (IPPP Grant PV025/2011B and UMRG grant RP025-2012A).

REFERENCES

Abdul-baki AA, Anderson JD (1970). Viability and leaching of sugars from germinating barley. *Crop Sci.,* 10: 31-34.

Anon A (1982). The future of sainfoin in British agriculture. In: Proceedings of Meeting at Grassland Research Institute, Hurley, Grassland Research Institute, Hurley, pg. 2-15.

Aufrère J, Dudilieu M, Poncet C (2008). *In vivo* and in situ measurements of the digestive characteristics of sainfoin in comparison with lucerne fed to sheep as fresh forages at two growth stages and as hay. *Anim.,* 2 (9): 1331-1339.

Delgado M (2008). La esparceta o pipirigallo. Informaciones tecnicas N° 201.

Frame J, Charlton JFL, Laidlaw AS (1998). Temperate forage legumes. CAB International. Wallingford. 327 pp.

Frase MD, Fychan R, Jones R (2000). Voluntary intake, digestibility and nitrogen utilization by sheep fed ensiled forage legumes. *Grass Forage Sci.,* 55 (3): 271-279

Harris D (1996). The effect of manure, genotype, seed priming seed depth, and date of sowing on the emergence and early growth of *Sorghum bicolor* L., Moench in semi-arid Botswana. *Soil Till. Res.* 40: 73- 88.

Harris D, Tripathi RS, Joshi A (2000). On-farm seed priming to improve crop establishment and yield in direct-seeded rice, in IRRI: International Workshop on Dry-seeded Rice Technology', held in Bangkok, 25-28. International Rice Research Institute, Manila, Philippines, 164 pp.

Jafari AA, Goodarzi A (2006). Genetic and relationships between performances, quality and agronomic traits in 72 populations of alfalfa (*Medicago sativa*). *J Genet Plant Breeding of pasture and forest* 14:215-230.

Koivisto JM, Lane GPF (2001). Sainfoin: worth another look. Royal Agricultural College on behalf of the BGS Forage Legumes Special Interest Group.

Mohajer S, Jafari AA, Taha RM (2011). Studies on seed and forage yield in 10 populations of

sainfoin (*Onobrychis sativa*) grown as spaced plants and swards, *J Food Agr Environ,* Vol.9 (1): 222-227.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiolgia Plantarum.* 15, 473-479.

Nakhjavan S, Bajolvand M, Jafari AA, Sepavand K (2011). Variation for Yield and Quality Traits in Populations of Sainfoin (*Onobrychis sativa*). *American-Eurasian J. Agric. & Environ. Sci.,* 10(3): 380-386.

Özcan S, Sevimay CS, Yıldız M, Sancak C, Özgen M (1996a). Prolific shoot regermination from immature embryo explants of sainfoin (*Onobrychis viciifolia* Scop.). Plant Cell Rep., 16: 200-203.

Özcan S, Yıldız M, Sancak C, Özgen M (1996b). Adventitious shoot regermination in sainfoin (*Onobrychis viciifolia* Scop). *Tr. J. Bot.,* 20: 497-501.

Özgen M, Özcan S, Sevimay CS, Sancak C, Yıldız M (1998). High Frequency adventitious shoot regermination in sainfoin. *Plant Cell Tiss. Org. Cult.,* 52: 205-208.

Saglam S (2010). Growth regulators effects on *in vitro* shoot regeneration on sainfoin (*Onobrychis sativa*), *Biotechnology & Biotechnological Equipment.* 24 (4), 2077-2079.

Sancak C (1999). *In vitro* micropropagation of Sainfoin (*Onobrychis viciifolia* Scop.), *Tr. J. of Botany,* 23: 133–136

Scharenberg A, Arrigo Y, Gutzwiller A, Soliva CR, Wyss U, Kreuzer M, Dohme F (2007). Palatability in sheep and *in vitro* nutritional value of dried and ensiled sainfoin (*Onobrychis viciifolia*) birdsfoot trefoil (*Lotus corniculatus*), and chicory (*Cichorium intybus*). *Archives of Animal Nutrition,* 61 (6): 481-496.

Smoliak S, Johnston A, Hanna MR (1972). Germination and seedling growth of alfalfa, sainfoin and cicer milkvetch. *Can J Plant Sci.,* 52, 757-762.

Tatl P, Cerci IH, Azman MA, Gurdogan F (2001). The effect on food intake and nutrient digestibility of ensiling by mixing whole crop barley at the boot stage with sainfoin in lambs. *Turk Veterinerlik ve Hayvanclk Dergisi,* 25 (4): 521-526.

Tosun M, Soya H, Celen F (1988). Effect of different cutting practices and row spacing on seed yield and other organic characteristics of Sainfoin (*Onobrychis viciaefolia* Scop). Ege-University Ziraat Facultesi Dergisi 25(1):184-194.

Turk M, Celik N (2006). Correlation and path coefficient analysis of seed yield components in the sainfoin. *J Biol Sci.,* 6:758-762.

Waghorn G (2008) Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production - progress and challenges. *Anim. Feed Sci. Technol.,* 147 (1/3): 116-139.