ADVENTITOUS SHOOT REGENERATION FROM MATURE AND IMMATURE EMBRYOS OF PAPER SHELLED ALMOND ROOTSTOCK Cv. PARBHAT.

IQBAL H QADRI

Plant Tissue Culture Lab. Centre of Research for Development (C.O.R.D)

University of Kashmir, Srinagar, J&K, India-190006.

Abstract

Almond is one of the Rosaceous stone fruits of temperate crops. There is extreme variability among almond crop plants in terms of fruit size and taste due to cross pollination. The main of this study is to develop an effective protocol for conservation of this Root stock germplasm variability by using embryonal axis's under invitro regeneration system. Mature tissues of almond plant when used as explants for invitro regeneration are the most recalcitrant among stone fruits. Mature almond embryos from 40 day chilled Kernels after sterilization were cultured on MS (1/2) medium under the influence of BAP (0.5,2.0,2.5 and 3.0 µM) and IBA(1.0,2.5 and 5.0 µM) in presence or absence of GA_3 (0.5 µM). After 8 weeks of culture period the results were evaluated. Adventitious shoot regeneration was observed at BAP (2.0,2.5 and 3.0 µM) and IBA(1.0 µM) combination. Highest regeneration (62.22%) with average shoot number of 8 per explant was observed at BAP (2.5 µM) and IBA(1.0 µM). Addition of GA_3 (0.5 µM) significantly decreased the regeneration frequency (51.11%), with average shoot number of 2 per explant, at this concentration. In another study embryos from immature nuts without chilling period were cultured on BAP (2.5,5.0,7.5 and 10.0 µM) and IBA(2.5 and 10.0 µM) combination and after 8 weeks of culture period adventurous shoot regeneration was observed at BAP (5.0,7.5 and 10.0 µM) and IBA(2.5 and 5.0 µM) combination. Highest adventitious shoot regeneration (55%) with average shoot number of 12 per explant was recorded at BAP (7.5 µM) and IBA(2.5 µM) Combination.

Abbreviations: **BAP** -6-benzyle aminopurine; **IBA** — Indole-3-butyric acid;
GA_3—Gibberellic acid **MS**- Murashige & Skoog;

Key words: Almond; embryo; regeneration.

Introduction

Almond (*Prunus dulcis* Mill) belongs to family rosaceae and is one of the valuable crops of temperate region. The conventional method of almond propagation is by nursery plant budding technique (Hartman et al.1997) to conserve the unique characteristics of the cultivars. Another conventional method of propagation is seed grown plant, in which one plant is produced per seed and result in extreme fruit variability due to cross pollination. In order to conserve the highly priced rootstock germplasm in areas with little seed availability there arise a need to incorporate some non conventional methods of almond breeding which will help in raising almond rootstock at

large scale from little germplasm in relatively shorter period and irrespective of season. One such non conventional method is invitro embryo culture, which has been put to exploitation by for propagation of some stone fruits (*Prunus spp.)* by a number of workers (Tuky 1933; pinto et,al 1990; Ramming 1990; Antonelli 1992; Burgos and Ledbetter 1993) Srinivasan and Scoorza 2007;Lininig et,al 2007; Nagaty 2012; Margirata and Antonio. 2012). Although some work on almond other than embryo culture has been reported (Mehra and Mehra 1974; Rugni and Verma 1982; Bouza 1997), but till date no work on embryo culture of almond on such lines has been carried out. The results of this study may contribute significantly in production of multiple plantlets from a single embryo which are to be used then for root stock purpose.

Materials and Methods

Preparation of explants:

Mature embryos from forty day chilled kernels at 4°C and the embryos from immature almond, of cv. Parbhat collected 100-120 days after pollination under study were excised out with the help of scalpel. Only the tagellum or the embryonal axise's were isolated by removing the surrounding cotyledonary tissues, washed three times with distilled water and were then subjected to chemical sterilization. Completely sterilized embryos were then inoculated on MS (½) basal medium (1962) vertically downwards under the influence of different growth regulators. After 8 weeks of culture period the observations were recorded.

Preparation of Culture Medium:

Ready made MS medium (Murashige & Skoog 1962) supplemented with sucrose (3.0% w/v) as carbon source and agar (0.7% w/v Agar-Agar, Sigma) as solidifying agent were used. Plant growth regulators BAP (0.5-10 µM) , IBA(1.0,2.5 and 5.0 µM) and GA_3 (0.5 µM) were added to basal medium prior to pH adjustment and autoclaving. The pH of the media was adjusted to 5.7 followed by autoclaving (120°C at 15 lb pressure for 20 minutes). Explants were transferred to light (3000 lux provided by cool white fluorescent tubes) for adventitious shoot initiation. The cultures were then maintained at 25±3°C with 16 h photoperiod(3000 lux). After 8 weeks of culture period the explants were examined for shoot production.

Statistical Analysis:

In a completely randomized design (CRD), the experiments were carried out three times in fifteen fold replication. The frequency of regeneration was calculated as the average percentage of explants differentiating into adventitious shoots and subjected to one way ANOVA. The means were separated according to least significant difference (LSD) at $P<0.05$ level. All means are represented with mean±SE after 8 weeks of culture period.

Results

Sterilization of Embryo:

Experiment No: 1- Effect of different sterilents on embryos of almond excised from mature and immature sterilized kernels.

Both mature and immature embryo from overnight distilled water soaked kernels were excised aseptically under laminar air flow hood and then sterilized by using either Mercuric chloride ($HgCl_2$) or Sodium hypochlorite (NaOCl) either separately or in combination. Various embryo sterilization trials carried out are presented in table 1. Out of various trials highest sterilization percentage (86.67) with maximum survival rate(100%) was achieved by soaking embryos in a solution containing $HgCl_2$ (0.05%) and NaOCl (1%) for 15 min. followed by 3 times rinsing with autoclaved double distilled water.

Experiment No: 2- Effect of BAP, IBA and GA_3 on mature embryonal axis's of almond

Embryonal axis's from 40 day chilled (4°C) kernels of almond cv. *Parbat*, without cotyledons were cultured on MS (½) medium supplemented with BAP (0.5, 2.0, 2.5 and 3.0µM) in combination with either IBA (1.0µM) or both IBA (1.0µM) and GA_3 (0.5µM each) respectively. and the data was recorded after 8 weeks of culture period. (Table.2).

No morphogenetic response of mature embryos was observed on MS (½) basal medium. However highest percentage (62.22) of shoot regeneration with average number of 8.33 shoots per explant was recorded on BAP (2.5µM) and IBA (1.0µM) combination.(Fig.1). Shoot regeneration percentage decreased (53.33) with average shoot number of 3 per explant on increasing the BAP concentration to 3.0µM (Fig.2), or decreasing it to 2.0 µM. When BAP (2.5µM) and IBA (1.0µM) combination was fortified with GA_3 (0.5µM), shoot regeneration percentage further decreased to (51.11) with average number of 3 shoots produced per explant (Fig. 3). However there was marked increase in shoot length. Shoot regeneration percentage of 46.66 with average adventitious shoot number of 1produced per explant was also observed under combined influence of BAP (3.0µM), IBA (1.0µM) and GA_3 (0.5µM). The micro shoots produced under different trials were subcultured without isolating them from each other on MS (½) basal medium for shoot elongation, on which each shoot elongated up to 1.5 cm in average.

Mature embryos from non-chilled kernels or kernels subjected to less than 40 day chilling period (15 and 30 days respectively) were also tried for adventitious shoot regeneration, but they showed poor response followed by complete necrosis of plumule within a period of two weeks either on MS (½) basal medium or MS (½) medium supplemented with GA_3(0.5µM).

Experiment No —3

Effect of BAP and IBA on Immature embryonal axis of almond cv. Parbat.

Embryonal axes excised from immature almonds cv. *Parbat,* 100-120 days after pollination were cultured on MS (½) medium supplemented with BAP (2.5, 5.0, 7.5 and 10µM) and IBA (2.5 and 5.0µM) and the data was recorded after 8 weeks (Table 3). Maximum percentage (55) of embryonal axes differentiated into adventitious shoots directly with an average shoot number of 12 per explant on BAP (7.5µM) and IBA (2.5µM) combination (Fig. 4). Adventitious shoot number of 11 was recorded on BAP (10.0µM) and IBA (2.5) combination. When IBA concentration was increased to 5.0µM and used in combination with BAP (2.5, 5.0, 7.5 and 10.0µM), nodular callus formation was observed, which later on differentiated into shoots.

A combination of BAP (2.5 and 5.0) and IBA(5.0µM) produced non regenerative callus (Fig.5). Regeneration percentage decreased by 20-25% at higher concentrations of BAP (7.5µM and 10 µM) in combination with IBA (5.0µM). (Fig.6). All these micro shoots produced invitro were subcultured on MS (½) basal medium without separating them from each other for elongation.

Discussion

In vitro embryo culture of almond was carried out to asses the regeneration potential in terms of multiple plantlet production from single embryo as rootstock in a relatively shorter period than required in nature. Pre treatment of *Prunus* seeds to 1-5^{0}C for 40 days was necessary for normal adventitous shoot regeneration (Ivanika and Pretova 1986 and Kester et,al 1986; San and yildrim 2009), because in absence of cold treatment embryos failed to regenerate, developed low vitality and crinkled leaves (Zagaja 1962). Similar findings were confirmed in the present study on Almond embryos, when subjected to less than 40 day chilling period failed to regenerate. In current study maximum embryo sterilization was achieved by using NaOCl (1%) and HgCl2 (0.05%) solution for 15 minutes, which can be ascribed to the fact that embryos being highly fragile and NaOCl in a concentration range of 1-10% is always recommended for such type of explants (Hammerschalag 1986; Norton and Norton 1986; Tabachnik and Kester 1977;Kester et,al 1986; Linning et,al. 2007; Manish et,al.2016;Eyob, KW 2017). However this suggestion is in contradiction to the observations of a number of workers who used only HgCl2 (1-2%) for different durations to sterilize different fragile tissues of *prunus* spp. (Tukey 1933; Zagaja 1962; Mehra and Mehra 1974; Rugni and Verma 1982; Hossini *et al.* (2010) and Muna *et al.* (1999))

The difference in in vitro regeneration of juvenile and mature plant material of almonds has been reported by Mehra and Mehra (1974) and Tabachnick and Kester (1977). A similar type of response was also observed in current studies on embryo culture of almonds. Present studies on embryo culture have shown that the production of adventitious shoots occur directly from embryonal axes of both immature and mature embryos of almond. Direct regeneration of adventitious shoots from embryos of other *Prunus* species like apricot (Pieterse,1989; Esitken *et al.*1999) and Peach (Schmidt and Ketzel, 1993) has been reported at BAP (4.4-8.8µM) and IAA (0.57-5.7µM) or 2,4-D (10µM). In current studies 62.22% of adventitious shoot regeneration was achieved at lower levels of BAP (2.5µM) and IBA (1.0µM)

combination and contradicts the studies on embryo culture of peach (Srinivasan and Scoorza, 2007; Nagaty, MA, 2012;) who reported adventitious shoot regeneration from different parts of immature embryos on BAP (0.5 µM)and 2,4-D(5 µM) combination or TDZ(3.6 µM) and IBA(2.5 µM) combination.

However the inclusion of GA_3 (0.5µM) in regeneration media significantly reduced the number and frequency of adventitious shoot regeneration, which is in consonance with the studies of Rizzo *et al.* (1998) on peach and Han-lixing *et al.* (1999); San and Yildrim, (2009). on cherry, where GA_3 induced normal development of embryos instead of adventitious shoot regeneration. The current study on embryo culture also reveals the production of adventitious shoots directly from immature embryos, with maximum efficiency on BA (7.5 and 10µM) in combination with IBA (2.5 and 5.0µM). Such findings contradict the results of earlier workers who reported indirect shoot regeneration from embryogenic calli by using TDZ/BAP/Kn (0.44, 4.4 and 20µM) in combination with 2,4-D (1 and 4.5) or NAA (1.35µM) [Goeffreda *et al.,* 1995 in apricot; Pieterse, 1989 in apricot; Schneider *et al.* , 1992 in peach; and Tang *et al.,* 2000 in cherry; Linning et,al 2007 in Plum: Margirata and Antonio, 2012 in Peach].

Present studies conducted on embryo culture of almond revealed that it takes 16 weeks, including 6 weeks of chilling period in production of 8-12 plantlets from each embryo irrespective of season. The same process in nature involves 27 weeks and results in production of only one plantlet from each embryo and further the process in nature is season dependent. Obviously the present technique saves 11 weeks of propagation time and is 8-12 times more efficient than natural process of almond breeding system. In case of immature embryos this protocol takes only 10 week's time to regenerate 11-12 adventitious shoots from a single embryo, because immature embryos do not required 40 day vernalization

Acknowledgement

The author wish to thank director Centre Of Research for Development (CORD), University of Kashmir and Head, Division of Pomology, Sher-e-Kashmir University of Agricultural Sciences and Technology Kashmir (SKUAST-K) India for providing necessary Plant material and other research facilities.

Table 1. *Effect of different sterilents on embryos of almond excised from mature and immature sterilized kernels. Data recorded after 4 weeks of culture period.*

S. No	*Sterilent*	*Concent -ration %age*	*Duration in min.*	*Percentage Response **		
				Survival	*Contamination*	*Sterilization*
1	$HgCl_2$	0.05	5	100	80.00	20.00
2	$HgCl_2$	0.1	5	100	58.33	41.67
3	$HgCl_2$	0.1	10	54.54	Zero	100
4	NaOCl	1.0	5	100	53.33	46.67
5	NaOCl	1.0	10	100	26.66	73.34
6	NaOCl	1.0	15	100	26.66	73.34
7	NaOCl (1ml) + $HgCl_2$ (0.05g)	-	15	100	13.33	86.67

* Fifteen replicates /treatment/experiment

Table.2 *Influence of BAP, IBA and GA_3 on adventitious shoot regeneration from mature embryos of almond cv. Parbat after 8 weeks on MS (½) medium (Values given are the mean of three replicates).*

S.No.	*BAP (µM)*	*IBA (µM)*	*GA_3 (µM)*	*Response* percentage*	*No. of ad. Shoots/explant x±S.E.*
1	0.5	1.0	-	-	-
2	2.0	1.0	-	51.11	2.66±0.08
3	2.5	1.0	-	62.22	8.33±0.06
4	3.0	1.0	-	53.33	3.00±0.01
5	0.5	1.0	0.5	-	-
6	2.0	1.0	0.5	48.88	1.66±0.06

7	2.5	1.0	0.5	51.11	1.66±0.07
8	3.0	1.0	0.5	46.66	3.33±0.07

* Fifteen replicates /treatment/experiment

Table. 3. Influence of BAP and IBA combination on in vitro regeneration from immature almond embryos cv. Parbat after 8 weeks on MS (½) medium. (Values given are the mean of three replicates).

S.No.	*BAP (μM)*	*IBA (μM)*	*Response* percentage*	*No. of explants x±S.E.*
1	2.5	2.5	-	-
2	5.0	2.5	40.00	1±0.00
3	7.5	2.5	55.00	12±0.03
4	10.0	2.5	45.00	11±0.0
5	2.5	5.0	40.00	Callus
6	5.0	5.0	30.00	Callus
7	7.5	5.0	25.00	7±0.05
8	10.0	5.0	20.00	5±0.06

* Fifteen replicates /treatment/experiment

Fig.1

Fig.2

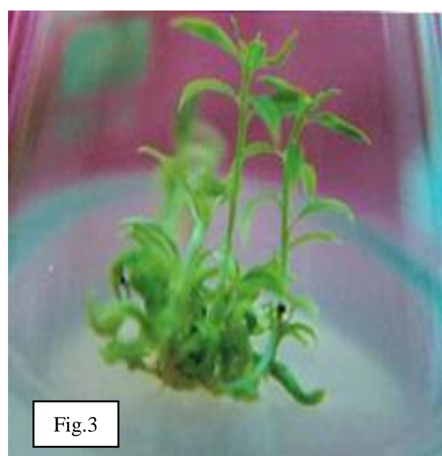
Fig.3

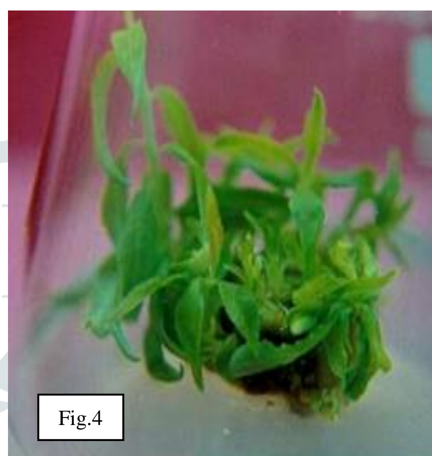
Fig.4

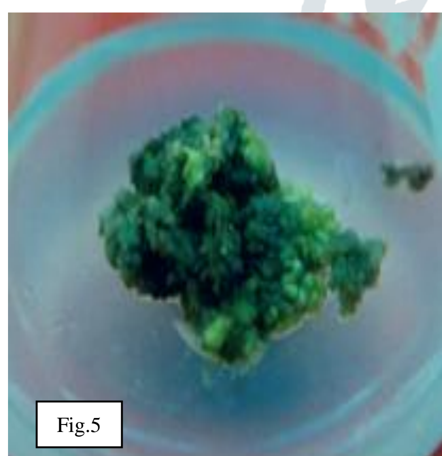
Fig.5

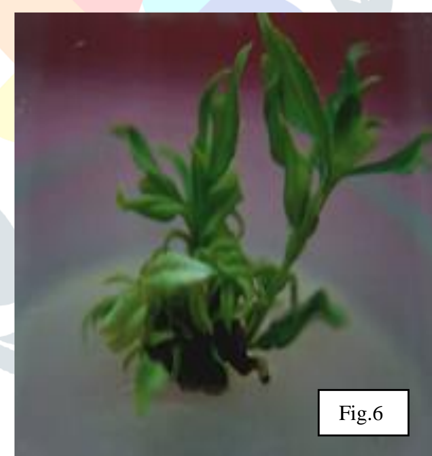
Fig.6

Legend:

Adventitous Shoot Regeneration from mature and immature embryos after 8 weeks.

Fig.1 Mature embryos on BAP (2.5µM), IBA (1.0µM).

Fig.2 Mature embryos on BAP (3.0µM), IBA (1.0µM).

Fig.3 Mature embryos on BAP (2.5µM), IBA (1.0µM) and GA_3 (0.5µM)

Fig.4 Immature embryos on BAP (7.5µM), IBA (2.5µM).

Fig.5 Immature embryos on BAP (2.5µM), IBA (5.0µM).

Fig.6 Immature embryos on BAP (7.5µM), IBA (5.0µM).

References:

Antonelli, M. 1992. Regeneration from almond cotyledons: induction of proembryonal masses. *Acta Horticulture* 300: 255-259.

Bouza, L. 1997. Micro propagation of *Prunus tenella* (Dwarf Russian Almond). In; Biotechnology in Agriculture and Forestry vol. 40. High-Tech. and Micropropagation VI (ed.) Y. P. S. Bajaj, pp: 276-288. Springer-Verlog Berlin Heidelberg.

Burgos, L and Ledbetter, C A 1993. Improved efficiency in apricot breeding: Effects of ebbryo development and nutrient media on invitro germination and seedling establishment .*Plant Cell Tissue and Organ Culture*.35(3):217-222.

Eyob,K W **2017**. Surface sterilization and invitro propagation of *Prunus domestica*.L Cv. stanley by using axillary buds as explants. Jr. *Biotech Res. 8: 18-26.*

Goffreda, J.C., Scopel, A.L. and Fiola, J.A. 1995. Indole butyric acid induces regeneration of phenotypically normal apricot (*Prunus armeniaca* L.) Plants from immature embryos.

Plant Growth . Regulation. 17(1): 41-46.

Hammerschlag, F.A., Bauchan, G.R and Scorza, R. 1987. Factors influencing in vitro multiplication and rooting of peach cultivars. *Plant Cell Tissue and Organ Cultures* 8(3): 235-242

Han-Lixing, Zhao-Gairong, Li-Hongyu, Huang-Zhengguag, Li-Li, Han-Lx, Zhao, G.R., Li-H.Y., Huang, Z.G. and Li, L. 1999. Embryo culture of early sweet cherry varieties. *China- Fruits*. 1: 28.

Hartman, H.T., Kester, D.E., Davies, F.T. and Geneve, R. L. 1997. Plant Propagation, Principles and Practices. Prentice Hall of India , New Delhi.

Hossini, AD., Moghadam, EG. And Anahid, S. 2010. Effects of media cultures and plant growth regulators in micropropagation of Gisela 6 rootstock.*Annals of Biological Research,* 1: 135-141.

Ivanicka, J and Pretova, A. 1986. Cherry (*Prunus avium* L.) In; Biotechnology in agriculture and Forestry vol. I. (ed.) Y. P. S. Bajaj. pp: 154-169.Springer-Verlag, Berlin Heidelberg.

Kester, D.E., Liu, F., Fenton, C.A. L. and Durzan, D.J. 1986. Almond (*Prunus duleis* (Miller) D.A. Webb). In; Biotechnology in Agriculture and Forestry vol. I. (ed) Y.P.S. Bajaj. . pp: 414-430. Springer-Verlag, Berlin Heidelberg.

Lining T & Yan W & Jayasankar, S & Sibbald,S (2007) Regeneration of Prunus salicina Lindl (Japanese plum) from hypocotyls of mature seeds *In Vitro Cell. Dev. Biol.-Plant* (2007) 43:343–347.

Manish T, Vishal S, Dharma P S, Garima K and Manu V. **2016**.Invitro propagation of virus indexed Gisela-5 (*Prunus cerasus x Prunus canescense*) clonal cherry rootstock. *Int. Jr crop Sc. 2(2):87-99*

Margarita P, Antonio C N (2012) Regeneration of peach (Prunus persica L. Batsch) cultivars and Prunus persica 3 Prunus dulcis rootstocks via organogenesis. *Plant Cell Tiss Organ Cult* (2012) 108:55–62

Mehra, A. and Mehra, P.N. 1974. Organogenesis and plantlet formation in-vitro in Almond. *Bot. Gazz.* 135(1): 61-73.

Muna, AS., Ahmad, AK., Mahmoud, K. and Rahman, KA. 1999. *In vitro* propagation of a semi-dwarfing cherry rootstock. *Plant Cell, Tissue and Organ Culture,* 59: 203-208.

Murashige, T. and Skooz, F 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant*. 15: 473-497.

Nagaty M A (2012) Establishment of Regeneration system for Taif peach (*Prunus persica* L. Batsch) cultivar (Balady cultivar) in Taif, KSA. *Journal of American Science*, 2012;8(4):232-239

Norton, M.E. Nortan, C. R. 1986. Explant origin as determinant of in vitro shoot proliferation in *Prunus* and *Spiraea. Journal of Horticultural Sci.* 61(1), 43-48.

Pieterse, R. E. 1989. Regeneration of plants from callus and embryos of 'Royal' apricot. *Plant Cell tissue and Organ Culture*. 19(2): 175-179.

Pinto, A. C. Q. Bryne, D. H. and Rogers, S. H. D. 1990. Influence of in-vitro conditions on growth of immature embryos of peach(*P. persica* L. Batsch). *Hortscience* 25(9): 98.

Ramming,D W 1990.The use of embryo culture in fruit breeding.*Hortscience*. 25(4): 393-398.

Rugini E and Verma D. 1982. Micropropagation of difficult to propagate Almond (*P. amygdalus*, Batsch) cultivar. *Plant. Sci. Letters* 28,273-281.

Ruzza, M. Bassi, D. Bryne, D. Porter, K and Monet, R 1998. Growth of immature peach (Prunus persica) embryos on different media. *Acta Horticulture*. 465: 141-144

San, B. and Yildirim, A.N. (2009), Seed and *in vitro* embryo germination in aged almond *Seed Sci. & Technol.,* **37**: 365-371

Schneider, K. E. Speranzini, D. and Biggs, A. R. 1992. Ontogeny of shoot regenerants on excised immature peach embryos. *Canadian Journal of Plant Science*. 72(2): 497-506

Srinivasan, C and Scorza, R (2007). The Influence of Genotype on the Induction of Somatic Embryos from InVitro Cultured Zygotic Embryos and Adventitious Shoot Regeneration from Cotyledons of Peach and Nectarine *Proc. IS on Biotechnol. Temp. Fruit Crops & Trop. Species* Eds. R.E. Litz and R. Scorza Acta Hort. 738, ISHS 2007 pp: 691- 96

Tang, H.R., Ren, Z.L., Krezal, G., Tang, H.R. and Ren, Z.L. 2000. Somatic embryogenesis and Organogenesis from immature embryo cotyledons of three sour cherry cultivars (*P. cerasus* L) *Scientia Horticulturar* **83(2);** 109-126.

Tukey, H. B. 1933. Growth of peach embryo in relation to growth of fruit and season of ripening. *Proc. Soc. Hort. Sci.* 30,71.

Zagaja, S.W. 1982. The effect of Gibberellic acid on cherry seedling growth. *Hortic Res.***1;** 81-84.