green berries, which blacken with maturity, and are toxic [Baker 1875].

Wild relative species in the genus *Asparagus* have many agricultural traits, such as salt tolerance, drought tolerance, acid soil tolerance and disease resistance. Among others *A. setaceus* is resistant to *Stemphyllum* leaf spot [Bansal et al. 1986] and rust caused by *Puccinia asparagi* [Kahn et al. 1952 cit. after Kanno and Yokoyama 2011]. Some researchers reported interspecific hand-pollinated crossing among *A. officinalis* × *A. setaceus* [Kahn et al. 1952 cit. after Kanno and Yokoyama 2011] and *A. setaceus* × *A. densiflorus* [McCollum 1988] but unsuccessful.

Asparagus setaceus (Knuth) Jessop is cultivated as an ornamental plant in two cultivars 'Pyramidalis' (lose pyramidalis habitas) and 'Nanus' (dwarf habitas) and is the popular addition to unusual combination of bouquets. It is propagated by seeds – but this method is inefficient and vegetative propagation has proven slow and practically it is impossible on a large scale [Yang 1977]. Therefore we are looking a more sophisticated methods e. g. *in vitro* culture which offers an unlimited production of clones and gives possibility to transfer and express in plant genes from any sources. *A. officinalis* was the first plant to be regenerated from tissue culture in monocotyledonous plants [Loo 1945, 1946]. In comparison to numerous works on application of biotechnological methods for edible asparagus for ornamental asparagus reports are scare. To date there have only been a few reports on micropropagation of *A. setaceus* syn. *A. plumosus* [Ghosh and Sen 1994 cit. after Regalado et al. 2015, Fonnesbech et al. 1977 a, b, Pindel 2000, Regalado et al. 2015].

The paper presents results of research on the effectiveness of different chemical and physical factors for organogenesis and callus proliferation of *A. setaceus* 'Pyramidalis'. To my knowledge, this is the first report for *in vitro* culture of this cultivar, therefore preliminary studies were performed in which the kind of explant, types and concentrations of auxins and cytokinins (used separately or together in a medium) were tested. Effect of sucrose concentration, addition of ancymidol and light or dark conditions on differentiation processes has also been studied. Based on these results, was to establish an efficient *in vitro* shoot culture of *A. setaceus* 'Pyramidalis'.

MATERIALS AND METHODS

Culture media and conditions

In preliminary studies the basal media MS [Murashige and Skoog 1962] or LS [Linsmayer and Skoog 1965], types and concentration of growth regulators – auxins: IAA (3-Indoleacetic acid), 2,4-D (2,4-dichlo-rophenoxyacetic acid), NAA (1-naphtaleneacetic acid), Dicamba (3,6-dichloro-2--methoxybenzoic acid), IBA (indole-3-butyric acid), Picloram (4-amino-3,5,6-trichloropyridine-2-carboxylic acid) and cytokinins: kinetin (6-furfuryladenine), BA (6-benzy- laminopurine), adenine sulphate salt (6-aminopuryne sulphate salt), TDZ (thidiazuron) – separately and together. All media was supplemented with three different concentration of sucrose and of ancymidol (α-cyclopropyl-α-(4-mthoxy- phenyl)-5-pyrimidinene- thanol). The effect of light or darkness and kind of gelling agents (Difco agar or Phytagel™) on spears and root explants were likewise tested (tab. 1). Results of these experiments were used to determine the best conditions of *in vitro* propagation of *Asparagu setaceus* Kunth (Jessop) 'Pyramidalis' nodal explants (as described below). Evocative scoring method was used. Depending on the response to the agent (factors) assigned the appropriate number of points (the explanation given in Table 1).

Based on the results of pilot studies the variants of the highest scoring were selected. For caulogenesis were chosen MS medium supplemented with IAA (1.71 μM) – MI, IAA (1.71 μM) + BA (13.32 μM) that is, the weight ratio of auxin to cytokinin as 1 to 10 – MII, both with addition 88 mM sucrose, 0.8% of Difco agar and light conditions. The rooting media were supplemented with IBA (2.46 μM) without – M1 or with addition of ancymidol (2.88 μM) – M2. Media for callus initiation contained 2,4-D (9.05 μM) – MA, 2,4-D (9.05 μM) + adenine sulphate (10.86 μM) – MB and TDZ (9.08 μM) + adenine sulphate (10.86 μM) – MC and as gelling agent Phytagel™. This part of experiment was conducted in the dark.

Nodal culture

This experiments was carried out using young stems (know as spears) as explants. Nodal segments

Table 1. The morphogenetical response of *A. setaceus* 'Pyramidalis' to different chemical and physical factorsfor *in vitro* MS or LS medium

Explant/Factor	Concentration in MS/LS mediumμM	Process	Number of points*/ selectedsymbol
Ex vivo spears (nodes/internodes)			
Auxins			
IAA	1.71	caulogenesis	2 / MI
2,4-D	4.52; 9.05	callogenesis	3 / MA
NAA	0.54; 2.69; 5.37	rhizogenesis	0.5
Dicamba	4.52; 22.62; 45.25	callogenesis	0.5
IBA	2.46	rhizogenesis	1 / M1
Picloram	8.28	vary weak callogenesis	0
Cytokinins			
Kinetin	4.65	caulogenesis – initiation stage	0.5
BA	0.88; 4.44; 13.32; 17.74	caulogenesis	2 (at a concentration 17.74)
Adenine sulphate	10.86	improves callus phenotype	2
TDZ	9.08	the higher % of callogenesis, but callus hard and compact	1
Auxin + cytokinin			
IAA + Kinetin	1.71 + 4.65	caulogenesis	1
IAA + BA	1.71 + 0.88	caulogenesis	1
	1.71 + 4.44	caulogenesis	1
	1.71 + 13.32	caulogenesis	3 / MII
	1.71 + 17.74	caulogenesis	2
2,4-D + adenine sulphate	4.52 + 10.86	callogenesis	2
	9.05 + 10.86	callogenesis	3 / MB
NAA + kinetin	0.54 + 4.65	rhizogenesis	0.5
	2.69 + 4.65	rhizogenesis	0.5
	5.37 + 4.65	rhizogenesis	0.5
NAA + BA	2.69 + 17.74	rhizogenesis	0.5
	5.37 + 4.44	rhizogenesis	0.5
TDZ + adenine sulphate	9.08 + 10.86	callogenesis	2 / MC
Ancymidol	2.88	deformation of explants (in the initiation medium)	0
		shortening the time of rhizogenesis (in the rooting medium)	1
IBA + ancymidol	2.46 + 2.88		1 / M2

Table 1. Cont.

Sucrose	2% (59 mM)	callogenesis	1
	3% (88 mM)	organogenesis and callogenesis	1
	4% (118 mM)	magnification of explants and initiation of abnormal distorted shoots	0
Light/darkness		callus formation and further growth was stimulated by darkness	3
		shoots were initiated on the light	2
Agar Difco	0.8%	organogenesis (shoots than roots)	2
		weaker effect of callogenesis	1
Phytagel™	0.3%	development ofembriogenic callus	2
Roots (2–3 mm transverse slices)		lack ofmorphogenetical responses to all tested chemical and physical factors	0

* evocative score:
0 – no morphogenetical response (no reaction)
0.5 – weak reaction (single explants with shoots, roots or callus)
1 – single shoots, roots or callus initiation at approximately 50% of explants
2 – shoots, roots or callus in over 50% of explants
3 – more than 1 shoot or root and intensively growing callus on most of the explants

(ca 2 mm long) were excised from 6–8 cm long spears of *A. setaceus* 'Pyramidalis' plants growing in a greenhouse (primary explant). Spears were surface sterilized in 70% (v/v) ethanol, followed by immersion in 0.1% solution of $HgCl_2$ for 2–3 min and washed with sterile distilled water five times. Five explants were placed horizontally on the tested media (MI and MII) in each of ten Erlenmeyer flask and cultured in light conditions (80 μmol $m^{-2} \cdot s^{-1}$ provided by fluorescent light in a 16/8 h photoperiod) at 22°C. Every four weeks, explants in the aggregate were passaged on fresh medium. Healthy, 3–4 cm long excised shoots were transferred into rooting medium (M1 and M2). For acclimatization regenerated plantlets were removed from culture vessels and their roots were washed of the attached medium in running water and transferred to pots containing sterile soil (peaetmoss and perlite 1:1) and maintained in a humid (ca 80 %) and shady environment.

Callus induction

In this part of experiments two kinds of explants were taken from juvenile (*in vitro* regenerated shoots – secondary explants) and from *in vivo* (in greenhouse) growing plants (primary explants). The internodes were cut vertically into 1–2 mm thick slices and cultured on callus induction media (MA, MB and MC) in the dark conditions. Explants were transferred into fresh media every 4 weeks.

For each treatment five replications were prepared, one replication consisted of four Erlenmeyer flask (five explants were placed in each of vessel). Only the results of the second phase of experiments were verified statistically with the Student's t-test (significance level at $\alpha = 0.05$).

RESULTS AND DISCUSSION

Table 1 summarized the preliminary studies results which were conducted for selection of explants and culture conditions used in the following experiment. Data in the tab. 1 indicate that only shoots explants of *A. setaceus* 'Pyramidalis' showed morphogenetical response, explants from roots failed – did not proliferate in any of the combinations used throughout the experiment. It be noted that were no significant differences between MS and LS media components (data not shown). IAA, BA and Kinetin

singly stimulated shoot formation, but the combination IAA with BA was found to be most effective for shoots production. The choice of the source of the explant from the mother plant of *Asparagus* species is relatively simple and is determined according to the goals. *Asparagus* belonging to the sclerophytes show a reduction in evaporating surface area, they are leafless and have spherical stems modified to perform the functions normally ascribed to leaves – cladodes. Shoots (spears) can be a source of adventitious buds (nodal explants), parenchyma tissue ability to the regenerate buds and roots directly or indirectly via callus. Comparative anatomy of stem and root of some *Asparagus* species [Nawaz et al. 2012] have shown significant differences among ground tissue, sub-epidermal layers, vascular bundles and sclerenchyma tissue among all species. This results in other response different genotypes to *in vitro* conditions. Already Rastogi and Sawheny [1989] noted that various factors in *in vitro* cultures (e.g. PGR levels and ratios, nutritional requirements, temperature and light) must be studied separately for each given species or even cultivar. The preliminary experiments showed that for rooting of regenerated shoots auxin IBA was the best and the addition of ancymidol reduced time of rooting. Callus tissue proliferated on the media supplemented with auxin 2,4-D or cytokinin TDZ, and addition of adenine sulphate improve callus phenotype. *A. setaceus* is highly salt tolerant [Bezona et al. 2009] and height concentration of nitrogen promoted their vegetative growth [Ziv and Naor 2006] but any changes in other components lead to differences in morphogenetical response – which is confirmed by this study (as seen in table 1).

Nodal culture

Based on the results presented in Table 1 nodal culture were conducted on the selected media MI and MII. On the MI medium the nodes have shown single and on MII multiple shoot formation (fig. 1). These shoots on subculture grew and branched. IAA singly (MI) showed week multiplication coefficient (average 1–2 shoots per node, phot. 1) whereas a combination of IAA and BA was found to be effective in multiplying adventitious shoots. This trend was observed up to four successive cultures when, after 16 weeks, 14 new shoots per explant were recorded. Similarly, Kar and Sen [1965] observed that IAA + BA in the ratio 1:10 was affected shoots development (12/expl) in *A. racemosus*. In contrary Pant and Joshi [2009] reported that NAA at many of the concentration (0.54, 2.69, 5.37, 10.74 μM) either singly or in combinations with cytokinins is effective in the shoot induction.

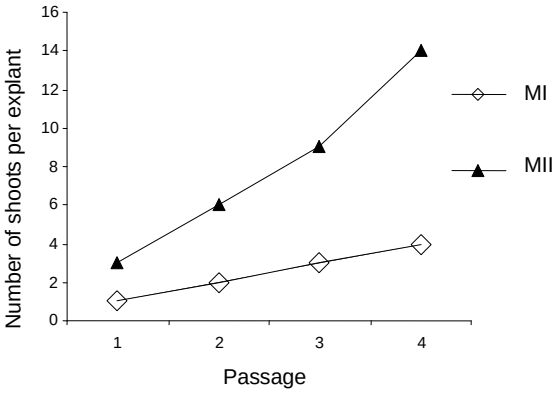
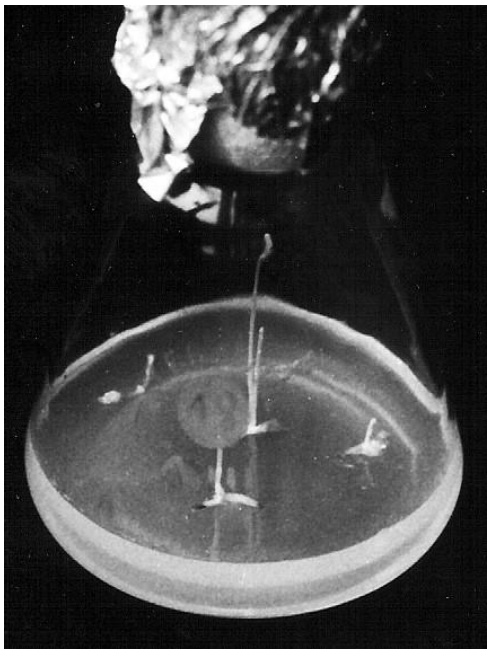


Fig. 1. The effect of growth regulators in MS medium on the number of shoots formation of nodal explants of *A. setaceus* 'Pyramidalis'.Medium MI contained: IAA (1.71 μM), MII: IAA (1.71 μM) + BA (13.32 μM)

The results of average number of roots on the excised microshoots and roots regeneration percentage (%) were given in Figure 2. As seen addition of ancymidol (in M2) increased rooting, almost twice, and number of roots per explant (to about 5). Ancymidol, a gibberelic acid synthesis inhibitor, has been used in culture medium to improve *A. officinalis* rooting frequency [Chang and Peng 1996], and somatic embryo production and germination [Li and Wolyn 1997, Ziv 2005]. Based on the preliminary study media was supplemented with 88 mM sucrose and 0.8% Difco agar. Present results are in agreement with those reported by Desjardins et al. [1987], Štajner et al. [2002] and Mehta and Subramanian [2005]. The mean number of roots on medium without ancymidol is similarly to present study (fig. 2).

Kar and Sen [1985] reported that root induction was supported by IAA (max. 4 roots/shoots) and Mehta and Subramanian [2005] by IBA + ancymidol (an average 5.7 roots/shoot) of *A. racemosus* and *A. adscedens* respectively. But in the present study the percentage of rooted shoots (ranged between 1 and 10 depending on the culture medium) was significantly lower than in the works of *A. racemosus* (70%) and *A. adscedens* (69%).



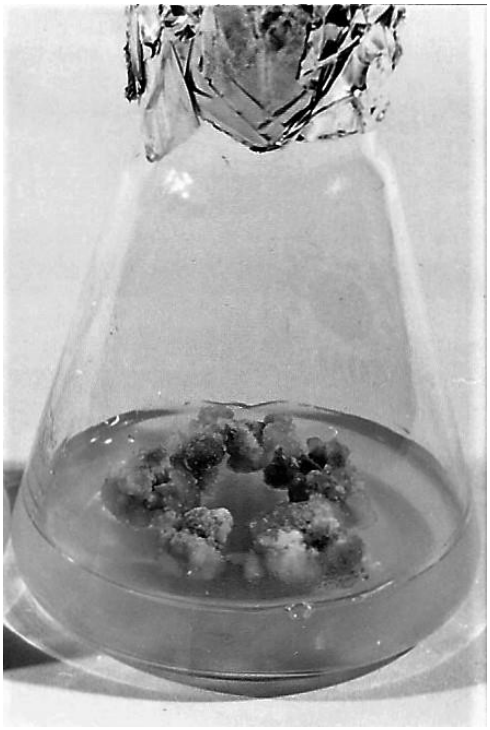
Phot. 1. Initiation of shoots from nodal explants on MS medium supplemented with 1.71 μM IAA (MI)

Rhizogenesis in asparagus is usually more difficult than caulogenesis and multiplication process. Several authors have pointed out that the major obstacle of Asparagus micropropagation protocols is root initiation [Sarabi and Almasi 2010]. Many genotypes are recalcitrant to adventitious root formations [Araki et al. 1996]. Ren et al. [2012] reported that among different factors affecting *in vitro* rooting, genotype and cultivar are of prime importance.

At the final developmental stage of acclimatization the successful gradually acclimatized percentage reached to 90% of rooted shoots. Similarly, high survival rate obtained for *A. recemosus*. Patel and Patel [2015] reported 75% survival rate in the field conditions and Kar and Sen [1985] 70% survival rate in pots containing sandy soil and humus.

Callus induction

To compare the *in vitro* responses of mature and juvenille tissue in this part of experiment primary (*ex vivo*) and secondary (*ex vitro*) material were taken. The selection of explant at a specific responsive stage is the important factor in overcoming in *in vitro* re calcitrance especially in perennial plants [Benson 2000]. Figure 3 show that in any cases secondary



Phot. 2. Close view of callus on MS medium supplemented with 9.08 μM TDZ and 10.86 μM adenine sulphate – MC (after 3 months of culture)

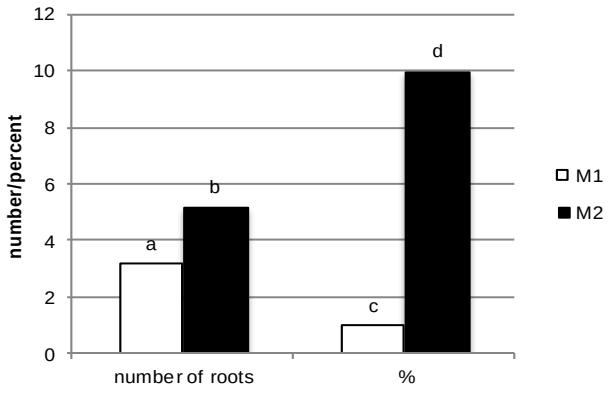


Fig. 2. The effect of ancymidol on rooting *A. setaceus*' Pyramidalis' shoots and onpercentage on rooted shoots. Medium M1 contained: IBA (2.46 μM), M2: IBA (2.46 μM) + ancymidol (2.88 μM)

(*ex vitro*) was suitable for callus initiation. A combination of TDZ and adenine sulphate (MC, phot. 2) found to be most suitable for faster growth of the callus tissue (135 mg), whereas 2,4-D is most effective in initiation callus culture from explants (89% explants with callus), but such combination (MA and MB) results in a low regeneration potential. However in many plant species application of 2,4-D proved to be highly efficient in the initiation of callus in monocot cultures, but inhibits further regeneration of the callus [Dasgupta et al. 2007, Pant and Joshi 2009]. So it was in the presented studies that examined genotype proved to be resistant to *in vitro* conditions. It is very importance to successful monocot tissue culture to select explants from meristematic regions. Monocotyledonous shoot meristems are basal in origin and stems which lack cambium tissue are especially culture-responsive [Benson 2000]. Asparagus stems contain parenchymateus cells in the vicinity of the vascular bundles and they undergo lignification if the stem allowed to grow [Rodrigez-Arcos et al. 2002]. As we can see phylogeny can have an immediate impact on tissue culture recalcitrance. These unpredictable *in vitro* reactions of *Asparagus setaceus* 'Pyramidalis' may be related to extreme xeromorphic habitats following phylloclade formations.

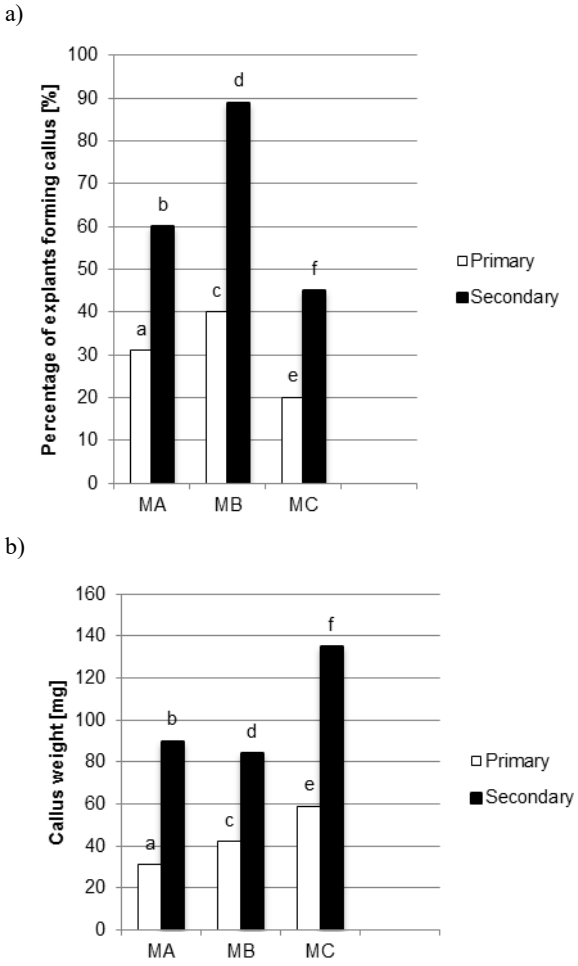


Fig. 3. The effect of origin of explants and medium on callus initiation (a) and mass (b) after 3 months of culture of *A. setaceus* 'Pyramidalis'. Medium MA contained 2,4-D (9.05 μM), MB contained 2,4-D (9.05 μM) + adenine sulphate (10.86 μM), MC contained TDZ (9.08 μM) + adenine sulphate (10.86 μM). Different letters indicate statistically significances at $\alpha = 0.05$

CONCLUSIONS

As described above, the present investigation showed that *in vitro* regeneration of *A. setaceus* 'Pyramidalis' can be obtained using nodal explants only. The final results of propagation by single-node cultures expressed as a ten starting shoots of donor plant: 6 nodes (or more) 14 new shoots 10% rooting

90% survival permitted to receive about 75 (or more) microplants. A high percentage of survival is probably due to the structural adaptations of asparagus to the dry environmental. On the other hand, a limiting factor in obtained a higher multiplications rate is sclerophyll features of asparagus plants.

ACKNOWLEDGMENT

This Research was financed by the Ministry of Science and Higher Education of the Republic of Poland

REFERENCES

Araki, H., Watanabe, S., Harada, T., Yakuwa, T. (1996). Aerial crown-like body formation through nodal segment culture in *Asparagus*. Proceedings VIII Int. Sym. on Asparagus. Acta Hort., 415, 209–214.

Baker, J.G. (1875). Flora of Australia online. ABRS, ©Commonwealth of Australia. 1994. Retrieved 2009-07-29.

Bansal, R.K., Menzies, S.A., Broadhurst, P.G. (1986). Screening of *Asparagus* species for resistance to *Stemphylium* leaf spot. New Zealand J. Agric. Res., 29, 539–545.

Benson, E. (2000). *In vitro* plant recalcitrance: an introduction. In Vitro Cell. Dev. Biol. Plant, 36, 141–148.

Bezona, N., Hensley, D., Yogi, J., Tavares, J., Rauch, F., Iwata, R., Kellison, M., Wong, M., Clifford, P. (2009). Salt and wind tolerance of landscape plants for Hawaii. Landscape, L–13, 1–9.

Chang, D.C., Peng, K.H. (1996). Phloroglucinol and tryptone enhance *in vitro* rooting and survival rate of asparagus nodal sections. Proceedings VIII Int. Sym. on Asparagus. Acta Hort., 415, 411–417.

Dahlgren. R.M.T., Clifford. H.T., Yeo. P.F. (1985). The families of the monocotyledons. Springer, Berlin.

Dasgupta, C.N., Mukhopadhyay, M.J., Mukhopadhyay, S. (2007). Somatic embryogenesis in *Asparagus densiflorus* (Kunth) Jessop cv Sprengeri. J. Plant Biochem. Biotech., 16(2), 145–149.

Desjardins Y., Tiessen H., Harney P., M., 1987. The effect of sucrose and ancymidol on the *in vitro* rooting of nodal section of Asparagus. HortScience, 22(1), 131–133.

Flora of Australia 1994. Online. ABRS, http://www.environment.gov.au/biodiversity/abrs/online-resources/flora/main/

Fonnesbech, A., Fonnesbech, M, Bredmose, N. (1977 a). Development of *Asparagus plumosus* shoot tips grown *in vitro*. Physiol. Plant., 40, 73–76.

Fonnesbech, A., Fonnesbech, M., Bredmose, N. (1977 b). Growth and development of *Asparagus plumosus* shoot tips *in vitro.* Acta Hort., 78, 287.

Kanno, A., Yokoyama, J. (2011). Wild crop relatives: Genome and breeding resources. Springer Verlag Berlin Heidelberg, 23–42.

Kar, D.K., Sen, S. (1985). Propagation of Asparagus racemosus through tissue culture. Plant Cell Tiss. Org. Cult., 5, 89–95.

Li, B., Wolyn, D.J. (1997). Interactions of ancymidol with sucrose and α-naphtaleneacetic acid in promoting asparagus (*Asparagus offcinalis* L.) somatic embryogenesis. Plant Cell Rep., 16, 879–883.

Linsmayer, E.M., Skoog, F. (1965). Organic growth factor requirements of tobacco cultures. Physiol. Plant., 18, 100–127.

Loo, S.W. (1945). Cultivation of excised stem tips of asparagus *in vitro*. Am. J. Bot., 32, 13–17.

Loo, S.W. (1946). Further experiments on the culture of excised asparagus stem tips *in vitro*. Am. J. Bot., 33, 156–159.

McCollum, G.D. (1988). *Asparagus densiflorus* cultivars Sprengeri and Meyers cross-pollinations with *A. officinalis* and other species. Asparag. Newsl., 6, 1–10.

Mehta, S.R., Subramanian, R.B. (2005). Direct *in vitro* propagation of *Asparagus adscendens* Roxb. Plant Tiss. Cult., 15(1), 25–32.

Murashige, T., Skoog, F. (1962). A revised medium for the rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15, 473–497.

Nawaz, T., Hameed, M., Waqar-U-Nisa, Ahmad, M.S.A., Younis, A., Kanwal, H. (2012). Comparative anatomy of root and stem of some native and exotic *Asparagus* L. species. Pak. J. Bot., 44, 153–158.

Pant, K.K., Joshi, S.D. (2009). *In vitro* Multiplication of wild Nepalese *Asparagus racemosus* through shoots and shoot induced callus cultures. Botany Res. Internat., 2, 88–93.

Patel, L.S., Patel, R.S. (2015). Rapid *in vitro* micro propagation of *Asparagus recemosus* Willd. from nodal explants. Int. J. Curr. Microbiol. App. Sci., 4(5), 607–617.

Pindel, A. (2000). Przydatność różnych metod *in vitro* w rozmnażaniu szparagów ozdobnych. Zesz. Nauk. AR Kraków, 266, 1–69.

Rastogi, R., Sawheny, V.K. (1989). *In vitro* development of angiosperms floral buds and organs. Plant Cell Tiss. Org. Cult., 16, 145–174.

Regalado, J.J., Carmona-Martin, E., Castro, P., Moreno, R., Gil, J., Encina, C.L. (2015). Micropropagation of wild species of the genus *Asparagus* L. and their interspecific hybrids with cultivated *A. officinalis* L., and verification of genetic stability using EST-SSRs. Plant Cell Tiss. Org. Cult., 121, 501–510.

Ren, J., Chen, W., Knaflewski, M. (2012). Factors affecting asparagus (*Asparagus officinalis* L.) root development *in vitro*. Acta Sci. Pol. Hortorum Cultus, 11(6), 107–118.

Rodriquez-Arcos, R.C., Smith, A.C., Waldron, K.W. (2002). Mechanical properties of green asparagus. J. Sci. Food Agric., 82, 293–300.

Sarabi, B., Almasi, K. (2010). Indirect organogenesis is useful for propagation of Iranian edible wild asparagus (*Asparagus officinalis* L.). Asian J. Agr. Sci., 2(2), 47–50.

Simpson, D.P. (1979). Casell's Latin Dictionary. London.

Štajner, N., Bohanec, B., Javornik, B. (2002). Genetic variability of economically important *Asparagus* species as revealed by genome size analysis and rDNAITS polymorphism. Plant Sci. doi: 1016/SO168-9452(02)00039-0

Yang, H.J. (1977). Tissue culture technique developed for asparagus propagation. HortSci., 12, 140.

Ziv, M. (2005). Simple bioreactors for mass propagation of plants. Plant Cell Tiss. Org. Cult., 81, 277–285.

Ziv, M., Naor, V. (2006). Flowering of geophytes *in vitro*. Prop. Ornam. Plants, 6, 3–16.