Evaluation of the Efficiency of Thidiazuron and Picloram for the Induction of Somatic Embryogenesis in Strawberry

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Abstract: The possibility of inducing somatic embryogenesis in petiole and leaf cultures of four cultivars of strawberry (*Fragaria* x *annanassa* (Duch)) using combinations of the cytokinin thidiazuron (TDZ) with the auxin Picloram was investigated. Explants were cultivated on MS medium (Murashige and Skoog 1962) containing different concentrations and combinations of TDZ (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg l^{-1}) and Picloram (2.0 and 4.0 mg l^{-1}). Regeneration of callus and shoots was achieved in different degrees in all cultivars, depending on Picloram and TDZ combinations. Shoots of different shapes and somatic embryo-like structures were observed. Histological examinations revealed that no somatic embryos in terms of bipolar structures were formed in response to any TDZ and Picloram combination tested, and that regenerants had to be classified as shoots and shoot-like or leaf-like structures.

Keywords: *Fragaria* x *ananasa* Duch, somatic embryogenesis, *in vitro.*

INTRODUCTION

Strawberry (*Fragaria* x *ananassa* Duch.) is one of the most popular soft fruits. Strawberries are produced in 71 countries worldwide on 506,000 acres and are among the highest-yielding fruit crops Husaini *et al.* 2008. Among the countries leading in strawberry production, Egypt is ranked fourth, with a production of 238, 432 tonnes in 2010 (FAOSTAT, 2012). To meet the demands of increasing strawberry cultivation, the strawberry industry in Egypt need to expand nursery production of certified transplants. However, this will not be achieved unless enough *in vitro* disease-indexed plants from nuclear stocks will be produced for further propagation in the nursery fields.

Tissue culture techniques provide suitable tools for clonal propagation of disease-free strawberry plants in a short time. *In vitro* mass propagation of strawberry is usually achieved through axillary shoot proliferation from pre-existing buds (Boxus, 1974, Swartz *et al.* 1981), but can also be performed through direct shoot regeneration from leaf disks (Mohamed *et al.*, 2007) or potentially through the formation of somatic embryos (Husaini *et al.* 2008).

In vitro propagation procedures need to be efficient, reliable and have to ensure genetic identity of the propagated plants. Meristem culture-derived plants of strawberry produce more runners per mother plant; give a higher yield per square meter but with a lower weight of mean and large fruits than runner-propagated plants (Swartz *et al.* 1981). Meristem culture-derived plants showed increased disease susceptibility against root-rotting fungi (Shoemaker *et al.*, 1985), abnormalities with multiple apices (Anderson *et al.* 1982) and more off-type leaf variants (Morozova, 2003) in each case dependent on cultivar. The process of meristem isolation from runner tips is time consuming and requires special skills. Due to labor-intensiveness of the cuttings and subculture operation, micropropagation by axillary-bud proliferation is costly (Litz and Gray, 1995). A propagation method based on the quite different way of regeneration by somatic embryogenesis could be a valuable alternative due to its high propagation rates (Neumann and Grieb, 1992) and the possibility of less somaclonal variation as it has been reported for Panicum maximum (Hanna *et al.*, 1984).

There are only a few publications on somatic embryogenesis in strawberry (Wang *et al.* 1984, Donnoli *et al.* 2001, Biswas *et al.* 2007, Husaini and Abdin 2007, Kordestani and Karami 2008, Husaini *et al.* 2008, Gerdakaneh *et al.* 2009, Gerdakaneh *et al.* 2011, Pallavi *et al.* 2011). All of them report the establishment of embryogenic cultures and the regeneration of structures which were classified as somatic embryos, but without presenting a convincing proof of somatic embryogenesis by histological means.

From these reports Husaini and Abdin (2007), Husaini *et al.* (2008), and Pallavi *et al.* (2011) used the cytokinin thidiazuron (TDZ) in different concentrations with different explant sources and genotypes.

Cytokinins such as kinetin, benzylamino purin (BAP) or thidiazuron (TDZ) have been useful in initiating somatic embryogenesis in several other plant species (Dunstan *et al*, 1995) and are usually supplied with an auxin in the induction medium. As reviewed by Raemakers *et al* (1995), BAP was most often used for somatic embryogenesis (57%) followed by kinetin (37%), zeatin (3%) and TDZ (3%). Nakano *et al* (2004) obtained the highest embryogenic callus from several *Tricyrtis spp* upon testing two auxins (2,4-D and Picloram) alone or in combination with TDZ. A combination of 4.5µM 2,4-D and 0.45 µM TDZ was most effective for inducing embryogenic callus.

The requirement of the combination of an auxin with a cytokinin for somatic embryogenesis induction was discussed by Pasternak *et al.* (2002). They mentioned that one of the possible targets of auxin action in this respect is the induction of expression of the cdc2 gene coding for the key regulatory protein kinase of the cell cycle. Auxin alone can result in the accumulation of this protein in high amount, but for the activation of kinase, the presence of cytokinin is required.

TDZ, a substituted phenylurea was shown to act as a substitute for both the auxin and the cytokinin requirements of organogenesis and somatic embryogenesis (Murthy *et al*, 1998).

Picloram, a chlorinated derivative of picolinic acid, was classified as an auxin suitable for the induction of somatic embryogenesis (Raemakers, 1995). Picloram was reported to induce embryogenic callus from roots of Oncidium (Wu *et al*, 2004), flower buds of carnation (Karami and Kordestani, 2007), young male flowers of banana (Yue-rong *et al*, 2004), shoot meristems of peach palm (Steinmacher, 2007) and nucellar callus of Cashew (Cardoza and Souza, 2002). Picloram induced somatic embryos from bulb-scales of *Lilium*-hybrids (Haensch 1996). However, Picloram was described as being not effective for the induction of somatic embryogenesis in other plants (Nakano *et al*, 2004; Kong *et al*, 2009; and Venkatesh *et al*, 2009). As studied by Raemakers (1995), among all auxins reported to induce somatic embryogenesis, Picloram represents only 5 %, indicating the limited use of this auxin in this process. In most, if not all of the above-mentioned reports, Picloram was supplied alone to medium designed for somatic embryogenesis.

The aim of this study is to examine the suitability of combinations of the cytokinin TDZ with the auxin Picloram for the induction of somatic embryogenesis in strawberry using regeneration experiments in vitro supplemented by histological examinations of the embryonic nature of the regenerating structures.

MATERIAL AND METHODS

Two explant types (leaf disk (5x5mm) and petiole (1.0 cm)) have been used from *in vitro* grown plants of strawberry (*Fragaria* x *ananassa* Duch., cv. Korona, cv. Chandler, cv. Tudla and cv. Sweet Charlie). Leaf disks and petioles were cultured on MS medium (Murashige and Skoog 1962) containing different concentrations and combinations of TDZ (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg l^{-1}) and Picloram (2.0 and 4.0 mg l^{-1}). Medium was supplemented with sucrose (30 g l^{-1}) and agar (7 g l^{-1}). The pH was adjusted to 5.7 before autoclaving (121°C, 15 min). Each treatment combination had three replications, each replication included 10 glass jars (ca 50 ml) containing 10 ml medium with one explant, per jar.

All cultures were incubated at 24°C with a 16 h photoperiod (69µmol $m^{-2}s^{-1}$) for 4 weeks. Callus was then transferred to another MS medium without plant growth regulators (PGR), supplemented with higher concentration of sucrose (80 g/l), agar (7 gl^{-1}) and the pH was adjusted to 5.8 before autoclaving (121°C, 15 min). All cultures were incubated for 4 weeks at the same conditions as mentioned above. The experiment was arranged in a factorial in completely randomized design. The data were recorded after 8 weeks using a stereo-microscope inside a laminar air flow cabinet and included the following *in vitro* parameters:

Percentage of responding explants, i.e. explants which showed visible growth of callus. and/or regenerating structures, number of somatic embryo like structures (SELS)/ explant and number of shoots/ explant.

Histological Evaluation

For histological analysis explants with regenerated structures were treated as described by Haensch (2004). Fixation was performed in a mixture of formalin, alcohol and acetic acid (FAA). A volume of 100 ml of this solution contained 5.4 ml formalin (37%), 65.6 ml ethanol (96%), 5 ml glacial acetic acid and 24 ml distilled water (Gerlach 1984).

After fixation the plant material has been embedded in hydroxyethylmethacrylate (Histo-technique-set Technovit 7100; Kulzer, Werheim, Germany). At the beginning specimens with representative structures were dehydrated in steps of 2h through a graded series of ethanol (70%, 90%, 96% and 100%), pre- infiltrated overnight with a solution of equal parts of 100 % ethanol and Technovit 7100 base liquid and then transferred into an infiltration solution of 100 ml Technovit 7100base liquid and 1 g hardener I for 1 day. At the start of the last two processes a vacuum was generated and kept for 30 min. For embedding the explants were put in Teflon molds with a mixture of 15 parts infiltration solution and one part hardener II. Then the explants were polymerized for 1 h at room temperature followed by further 6h at 37°C. Afterwards the samples were mounted on block- holders with Technovit 3040. Slices of 6 µm thickness were cut at room temperature using a Jung CM1800 microtome with type 818 disposable microtome blades (both from Leica Instruments, Nussloch, Germany). Slices were stretched on the surface of distilled water, mounted on slides and then stained with 0.05% toloudine blue O (Serva, Heidelberg, Germany). The latter was dissolved in 1% sodium tetraborate decahydrate buffer (Hutchinson *et al*. 1996a). The slices were rinsed using distilled water, dried and covered with Entellan (Merck, Darmstadt, Germany) and a cover slip. This procedure stains the cytoplasm and unlignified cell walls red and the DNA- containing structures and lignified cell walls blue (Gerlach 1984). Microscopic analysis was performed using a Axio Imager A1 microscope (Zeiss, Jena, Germany) equipped with an Axio Cam MRc 5 camera (Zeiss, Jena, Germany).

RESULTS

In Vitro Regeneration

Explants that remained unchanged or died were considered as non- responding. In total between 80 and 100% of the cultured explants responded to the culture treatments by the formation of callus, shoots or somatic embryo-like structures (SELS). The development of callus, shoot buds and somatic embryo- like structure were detected from both basal and distal sides of the segments. Callus formed most often from the cut surface of petiole and leaf disk explants. Callus tissues were mostly compact and ranged in their extent from slight to massive, depending on treatments.

The observations by stereo microscope did not show clear somatic embryos at any stage of development, except structures that resembled somatic embryos. These tiny structures were detected on the surface of the

formed callus from different zones of the explants. They were first globular-shaped and later on they appeared as leafy irregular forms were a root pole could be assumed but not clearly detected (Fig. 1b-c, 2a, c-d, 3a-b). An unambiguous classification of these structures was not possible without histological analysis. Therefore these structures were designated as "somatic embryo-like structures" (SELS) for the interim. Because of this difficulty in the classification of these structures statistics of the experimental data has been restricted to simple descriptive ones (Table 1). The average number of SELS per explant depends on genotype, explant type and the concentrations of TDZ and Picloram. The average number of SELS ranged from zero in different treatments to 16.33 at maximum in petiole cultures of the cultivar Tudla on medium with 1.5 mg l^{-1} TDZ and 4 mg l^{-1} Picloram.

Beside SELS different shoot regeneration patterns were observed on the cultured explants, ranging from small bud initials, shoot like structures and complete plantlets with well visible leaves. Shoot bud initials regenerated directly, in most cases, from the cut surface of the cultured petiole and leaf explants, or from the mid vein of the leaf disk. The average number of regenerated shoots was dependent on treatment (Table 1) and ranged from zero to 16.67 shoots per explant at maximum on petioles of cv. Tudla on a medium supplemented with 3 mg l-1 TDZ and 2 mg l^{-1} Picloram. The patterns of shoot regeneration are illustrated in Figures (1a; 2b and 3c).

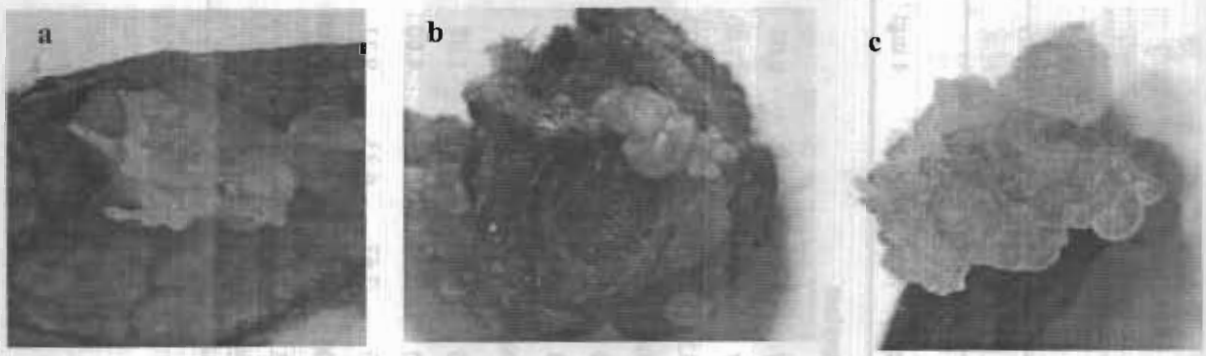

Figure 1. (a)Shoot bud regeneration from the mid vien of leaf explant on medium supplemented with 2.0 mgl^{-1} TDZ + 2.0 mgl^{-1} Picloram. (b,c) bud primordia and globular embryo- like structures arising for the cut surface of petiole explant on medium supplemented with 1.5 mgl^{-1}TDZ + 4.0 mgl^{-1} Picloram. cv.'Korona'

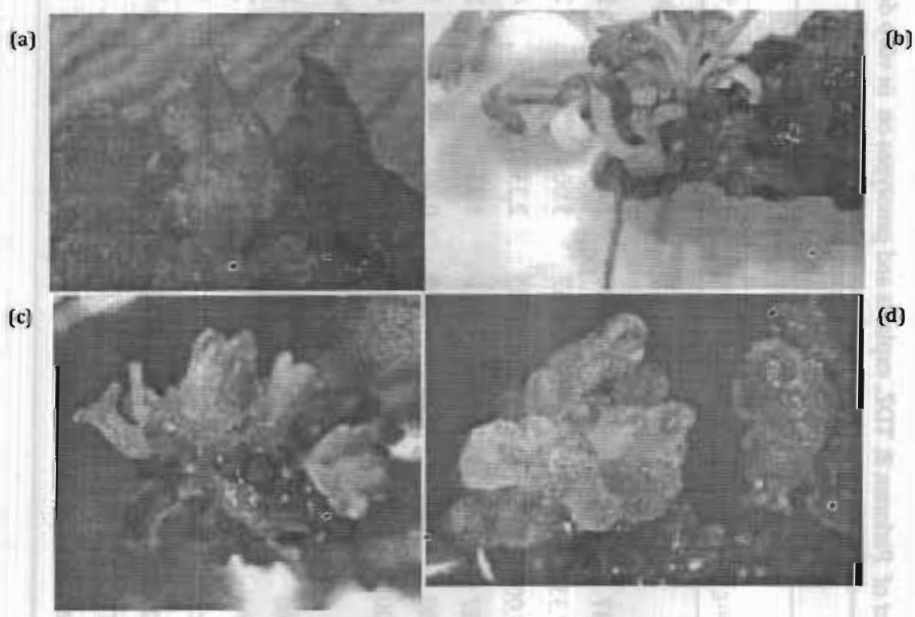

Figure 2. Morphogenic patterns of petiole (a,b) and leaf (c,d) explants of cv. Chandler showing the development of shoots and somatic embryo-like structures (SELS). (a) SELS (arrow). (b) Fully differentiated shoots, (c,d), SELS (arrows). Petioles of (a) and (b) are grown on MS + 2.0 mgl^{-1}TDZ +2.0mgl^{-1} picloram. Structures from leaf explant of (c) derived from MS medium + 2.0 mgl^{-1} TDZ +4.0 mg/l Picloram. SELS in (d) derived from MS medium + 2.0 mgl^{-1} TDZ +2.0 mgl^{-1}Picloram.

Table 1. Interaction Effect of Picloram & TDZ, explants and genotypes on *in vitro* development

Genotype	**Korona**				**Chandler**				**Tudla**				**Sweet Charlie**			
Explant	**leaf disk**		**petiole**		**leaf disk**		**petiole**		**leaf disk**		**petiole**		**leaf disk**		**petiole**	
Picloram	**2 mgl^{-1}**	**4 mgl^{-1}**	**2 mgl^{-1}**	**4 mgl^{-1}**	**2 mgl^{-1}**	**4 mgl^{-1}**	**2 mgl^{-1}**	**4 mgl^{-1}**	**2 mgl^{-1}**	**4 mgl^{-1}**	**2 mgl^{-1}**	**4 mgl^{-1}**	**2 mgl^{-1}**	**4 mgl^{-1}**	**2 mgl^{-1}**	**4 mgl^{-1}**
TDZ mg/l	Number of SELS/explant															
0.5	0.00	3.67	1.33	1.33	4.00	1.00	6.00k	5.00	2.67	1.67	2.00	0.00	6.33k	1.67	5.00	2.67
1	0.00	12.33	5.33	0.00	5.67	3.00	6.00k	2.67	1.67	6.67	2.00	13.00	10.33	6.67	8.33	1.67
1.5	6.00k	13.00	13.33	0.00	9.00	2.33	7.00	4.00	10.33	10.33	7.67	16.33	12.00	12.67	5.67	10.67
2	9.00	5.67	8.00	0.00	13.00	8.33	13.67	3.33	9.67k	10.67	9.33	15.67	7.67	14.33	5.67	5.00
2.5	7.00	0.00	5.33	0.00	8.00	0.00	3.33	1.67	0.00	1.33	2.33	4.33	8.00	2.00	9.33	0.00
3	0.00	0.00	0.00	0.00	1.67	0.00	5.00	2.00	3.33	0.00	3.67	0.00	3.67	0.00	0.00	0.00
	Shoots number/explant															
0.5	0.00	0.00	0.00	0.00	0.00	2.67	0.00	8.33	0.00	3.00	0.00	1.67	0.00	2.33	2.67	0.00
1	0.00	0.00	0.00	0.00	0.00	1.33	3.33	3.00	0.00	2.00	2.67	4.00	0.00	4.33	3.67	6.00
1.5	0.00	2.00	0.00	6.33	0.00	5.67	8.33	8.67	0.00	7.67	6.33	9.33	0.00	5.67	4.67	8.00
2	4.67	2.33	0.00	8.00	2.33	3.67	8.67	12.33	11.00	11.00	9.33	11.00	4.33	7.67	6.00	8.67
2.5	5.00	6.00	0.00	7.00	8.00	8.67	9.33	12.00	11.33	14.00	11.67	13.00	6.67	8.67	7.00	10.33
3	7.33	8.67	9.33	13.33	9.33	10.67	10.33	13.67	11.00	16.00	16.67	15.67	11.33	9.67	8.00	14.33

Figure 3. Shoot regeneration and somatic embryo-like structures (SELS) on leaf disk explants on MS +2.0 mgl^{-1} TDZ +4.0 mgl^{-1} picloram. (a) SELS (arrow) of cv. Sweet Charlie. (b) SELS (arrow) of cv. Tudla. (c) Shoots of cv. Tudla.

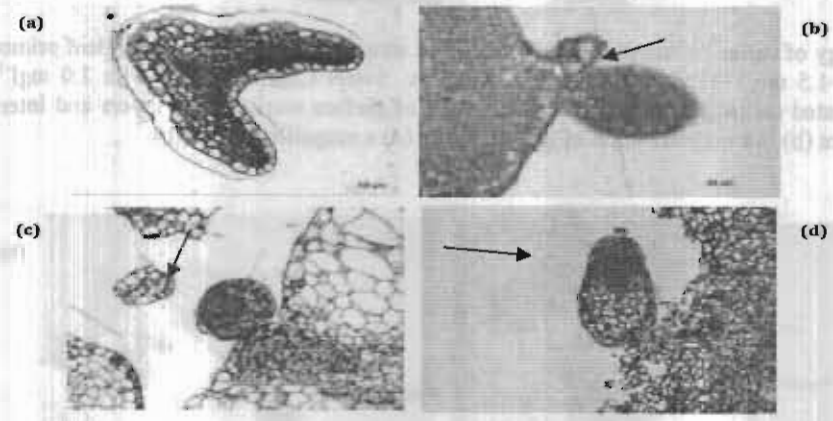

Figure 4. Histology of embryo-like structures. (a,b) Longitudinal sections through SELS of cv Korona from leaf-explant on medium supplemented with 2.0 mg l^{-1}TDZ and 2.0 mg l^{-1}picloram showing in (a) rather paranchymatous tissue with limited vascular strands. A root pole is not present. (b-d) Sections showing globular or oblong shaped embryo-like structures, which arose from the epidermal layer or from callus of a leaf disk explant. These structures show patially small cytoplasmically dense cells with a big nucleus and partially parenchymatous cells.

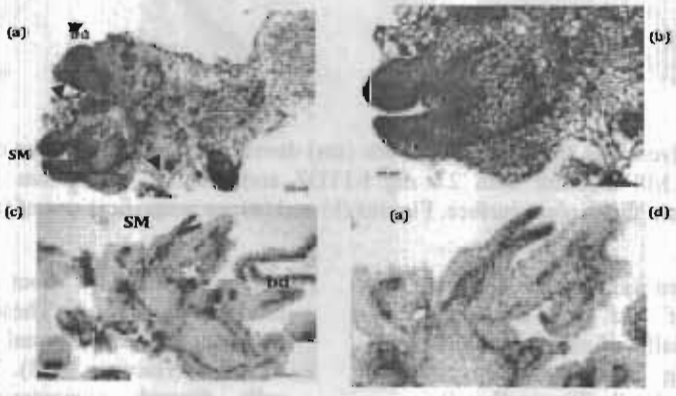

Figure 5. Histology of (a) shoot meristem (SM) and bud development (bd) from petiole cut surface of cv. Korona on medium amended with 1.5 mg l^{-1}TDZ +2.0 mg l^{-1}Picloram. Globular embryo –like structures (arrow) consisting of the same type of tissue like the buds. (b) Enlarged shoot meristem of (a) with two leaf primordia. (c) Section through the entire cultivated petiole of cv. Chandler on medium amended with 2.0 mg l^{-1}TDZ +2.0 mg l^{-1}picloram showing several shoot meristems and SELS with limited vascular strands.(d) enlarged section of (c).

Histological Exam nation

A structure closely resembling a somatic embryo was detected from a leaf explant on medium supplemented with 2.0 mg l^{-1} TDZ and 2.0 mg l^{-1} Picloram. The histological study revealed that the structure consisted mainly of paranchymatous tissue with limited vascular elements. A root pole was not detectable (Fig. 4a). Several small globular-shaped or

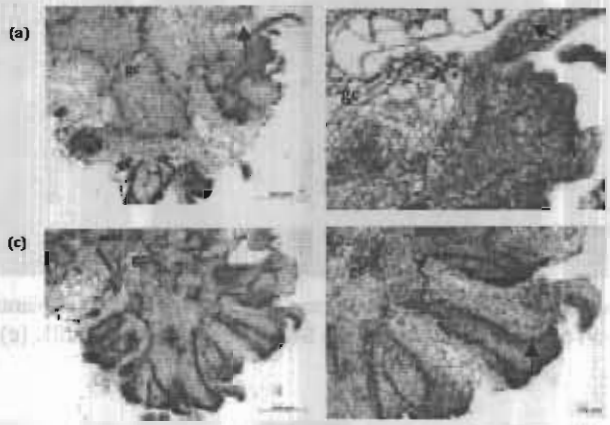

Figure 6. Histology of callus differentiation from leaf disk into multiple meristems with leaf primordia (arrows). a,b cv. Korona, on MS +1.5 mg l^{-1}TDZ .4 mgl^{-1}picloram; c,d cv. Sweet Charlie on MS with 2.0 mgl^{-1}TDZ and 4.0 mg l^{-1} picloram. Proliferated callus give rise to an organization of surface meristematic layers and internally located growth centers (gc). Figure (b) is a magnification of (a) and figure (d) a magnification of (c).

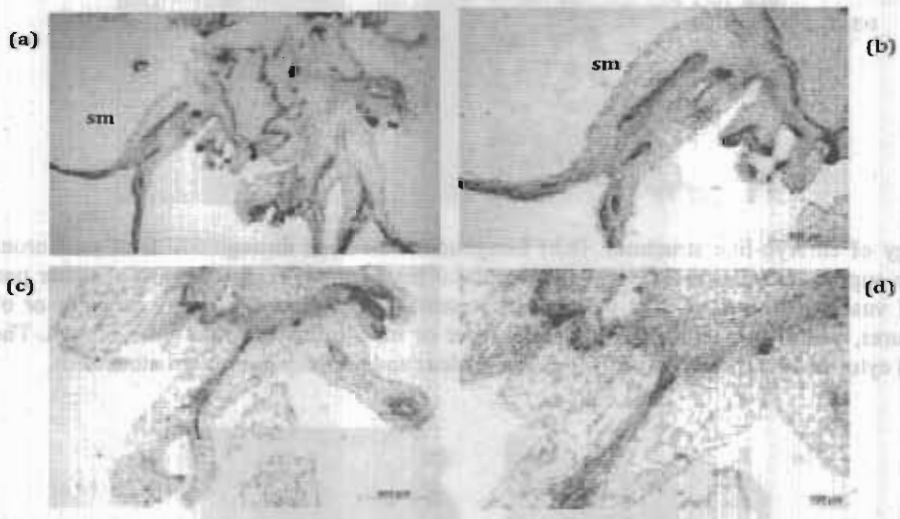

Figure 7. Histology of adventitius shoot meristem buds (sm) developed from leaf tissues of cv. Chandler (a,b) and cv. Tudla (c,d) cultured on MS medium with 2.0 mg l-ITDZ and 4.0 mg l-Ipicloram. Note the differentiation of meristematic structures from the explant surface. Figures (b) and (d) are magnifications of (a) and (c), respectively.

or oblong shaped structures were observed, arising from the epidermal layers of leaf disc explants. These structures consisted partially of small cytoplasmically dense cells with a big nucleus and partially of parenehymatous vacuolised cells (Figure 4b,c,d).

Shoot meristems and globular somatic embryo-like structures were induced from petioles of the cultivars Korona (Figure 5 a,b) and Chandler (Figure 5 c,d). These were connected with vascular strands to the explant (Figure 5d). The lack of a definite root pole in the aforementioned structures indicates that they are not somatic embryos.

DISCUSSION

Somatic embryos have been characterized as new individuals that differenciate during their further development both a shoot apex and opposite to this a discrete radicular end. These structures have no vascular connection to the original explant at any time during their life (Haccius 1978). They develop from somatic cells through characteristic embryological stages (Williams and Maheswaran 1986).

In terms of this definition the use of TDZ (Husaini and Abdin 2007, Husaini *et al.*, 2008) or Picloram (Kordestani and Karami 2008) alone in separate experiments with strawberry did not result in convincing proofs of their ability to generate somatic embryos in this species. Although the authors classified the regenerating structures as somatic embryos, in none of these examinations such bipolar structures have been shown by histological means. The results of the present examination are in accordance with this. Although the results showed that most of the used explantsresponded

to the applied treatments and that most of these treatments were suitable to give rise to regeneration in all cultivars (Table 1, Figures 1, 2, 3), the histological examinations of the regenerated structures clearly demonstrated that TDZ combined with picloram was only effective in the regeneration of shoot buds and shoots (Fig. 5, 6 and 7) or in structures that resemble somatic embryos only externally. These embryo-like structures show in their early stages characteristics comparable to somatic embryos, i.e. they are globular or heart shaped and consist at least partially of cells with a very dense cytoplasm with big nuclei which are typical for embryogenic cells (Fig. 4b-d, 5a,). Nevertheless, these early embryo-like structures did never develop to clear bipolar structures (Fig. 4a). The lack of a definite root pole in any of the obtained more developed structures revealed that these structures were not somatic embryos. This is in accordance with results of Haensch (2004) and Winkelmann *et al.* 2005 with regard to the use of TDZ in petiole cultures of *Pelargonium* x *hortorum*. No somatic embryos could be achieved, and the regenerants have been classified as shoots and leaf or shoot-like structures.

The use of TDZ in combination with Picloram for the induction of somatic embryogenesis in herbaceous species was very limited. In another report, picloram combined with other cytokinine types (BAP or Kinetin) gave rise to somatic embryogenesis from hypocotyle explants of green gram, a recalcitrant green legume (Kong and Aderkas 2007). Results of Sharma *et al* (2005) on wheat, and Sharma *et al* (2004) on barley did not indicate the induction of embryogenic callus, while the combination of 2.0 mgl-1 Picloram and 3.0 mgl-1 TDZ were found effective in inducing shoot regeneration from callus. Combination of TDZ and Picloram was also found to induce callus formation from corm sections of the lace plant, but no shoot regeneration was recorded (Carter *et al*, 2011). These examples are very contradictory and show that conditions which induce somatic embryogenesis in a certain species successfully may not be suitable in another one. Our results confirm the statement which has been made by Graham (2005) that the research on somatic embryogenesis in strawberry is still in a preliminary stage, and that more efforts are required to develop this kind of technology.

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إختبار كفاءة منظمي النمو TDZ و Picloram في بيئة مزارع الأنسجة على إحداث و تكوين الأجنة الجسمية في الفراولة

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**قسم الإكثار النباتي – معهد محاصيل الخضر و الزينة- ايرفورت – ألمانيا.

تم اختبار إمكانية إحداث الأجنة الجسمية من منفصلات نباتية للأوراق و أعناق الأوراق في أربعة أصناف من الفراولة و ذلك على بيئة MS محتوية على تركيزات مختلفة من منظم النمو TDZ(0.5- ١,٠ – ١,٥- ٢,٠- ٢,٥- ٣,٠ مجم/لتر) و Picloram (٢,٠- ٤,٠ مجم/لتر) حيث حدث تكشف للأنسجة المنزرعة الى كالوس أو تبرعم خضري بدرجات مختلفة اعتمادا" على تركيزات و تباديل مختلفة لكل من TDZ و Picloram في كل الأصناف. كما حدث تكوين للتبرعمات الخضرية في أشكال مختلفة، و كذلك تكوين تراكيب شبيهة بالأجنة الجسمية خاصة" من زراعة أنسجة الأوراق على بيئة تحتوي على ٢,٠ مجم/لتر TDZ و ٢,٠ مجم/لتر Picloram ، و قد أظهر الفحص التشريحي عدم تكوين أجنة جسمية حقيقية على أي من التركيزات و التباديل المختبرة لمنظمي النمو TDZ و Picloram و أنة يمكن اعتبار الأنسجة المتكشفة التي تم الحصول عليها على أنها أفرع خضرية أو شبيهة بالتبرعم الخضري أو الورقي.