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Thidiazuron Induced Plant Regeneration Via Organogenesis And Somatic Embryogenesis In Broccoli Brassica Oleracea Var. Italica

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Abstract

Broccoli, Brassica oleracea var.italica is an important plant due to its flavor and anticancer activity that received more attention in recent years. The leaf, stem and root explants of aseptic 15 day old seedlings, grown on MS medium were used for callus initiation.Theseexplants were cultured on agarsolidified MS medium containingBAP, NAA, TDZ plant growth regulators.Stem segments gave the highest rate of callusinduction that reached 98% on MS medium supplementedwith 0.2 mg L^{-1} NAA and 0.5 mg L^{-1}BAP. Also,callus had been initiated from root segments cultured on MS medium fortified with 1.5, 2.0 mg L^{-1} TDZ. The results indicated that shoot regeneration occurred after sub culturingthe callus in the same medium as for callus induction, in addition to MS medium supported with 4.0 mg L^{-1}BAP and 0.5 mg L^{-1} NAA. Moreover, different somatic embryos stages (globular, heart, torpedo and cotyledonary) were observed when stem callus wascultured on MS medium supplied with 0.5, 0.4 mg L^{-1} TDZ. I*n vitro* regenerated shoots derived from callus and those that developed from somatic embryos were transferred to MS medium free from growth regulators as well as MS medium containing 1.0, 2.0 mg L^{-1}IBA for rooting. Rooted plantlets have beensuccessfully acclimatized. An efficient approach was used for inducing shoot regeneration in broccoli Brassica oleracea var.italica via organogenesis and indirect somatic embryogenesis. This research proved that TDZwas effective in inducing somatic embryos from stem and root callus.

Key words: Thidiazuron, regeneration, broccoli, somatic embryogenesis.

Introduction

Brassica is an economically important genus, from the Brassicaceae family. Thisfamily contains many plants that are mostly used as crops for oilseeds, fruits, feed crops, and condiments(Gambhir and Srivastava, 2015; Farooq etal.,2019).Brassica oleraceaincludes the

Brassicacea's main vegetables and nearly every part of the plants was used; the leaves (cabbage and kale), flower buds (broccoli), terminal (early cauliflower), floral primordia (late cauliflower), axillary buds (Brussels sprout) and stem (kohlrabi) (Qin et al.,2006).Aforementioned plantsare commonly used as a valuable source of dietary fiber and have also been found to be useful in cancer prevention (Yussof etal.,2012; Mandrich and Caputo, 2020; Fahey and Kensler, 2021).

Broccoli,Brassica oleracea var.italica is a commercially important edible plant (Tilaar etal., 2012; Azis et al.,2015), due to its delicious flavor and anticarcinogenic properties, it is receiving more and more attention in recent years(Yuan et al., 2015; Zeng et al.,2017).It has a high content of vitamin A and C and calcium (Henzi et al., 2000; Yussof et al.,2012), and antioxidant including Indole-3-Carbinol, which prevents breast and colon cancer and work s to enhance liver function (Owis, 2015).The tumor rate incidence in esophagus and stomach can be decreased by regularly taking food that contains antioxidants. It was demonstrated that broccoli has a high benefit in daily consumption (FAO, 2017).

In vitro regeneration offers a chance to grow attractive and virtually genetically identical plants quickly(Farooqet al.,2019).Many factors influence this in vitro regeneration including the composition of the culture medium, source of the explants,cultural environment and genotype (Bano et al., 2010). The in vitro tissue culture technique is an essential experimental approach in applied research (Bednarek and Orłowska, 2019).Organogenesis is the most frequently recorded method of adventitious shooting in Brassica crops and different explants were used such as hypocotyles (Gerszberg et al., 2015),cotyledons (Munshi et al. 2007; Kamal et al., 2007), leaves (Gambhir et al., 2017), shoot tips (Widiyanto and Erytrina, 2001), and protoplasts (Chikkalaet al., 2009). In various brassica species, for instance Brassica napus,various factors such as combinations of plant regulators of growth, explant type and age were investigated for finding an efficient approach for high frequency regeneration of Brassica napus(Dina et al., 2019). The aim of this work is to obtain broccoli plants through organogenesis and somatic embryogenesisusing thidiazuron as an induction agent.

Materials and Methods

Seeds sterilization

Seeds of broccoli,Brassica oleracea var.italica, were washed with distilled water for 30 minutes, then surface sterilized via immersing in 70% ethyl alcohol for one minute (Pavlovic

et al. 2010), followed by submerging for 10 minutes in a commercial bleach solution(3% sodium hypochlorite NaOCl). The seeds were rinsed four times in sterile distilled water.

Callus initiation

Sterilized seeds were cultured in germination medium (Murashige and Skoog, 1962) containing 3% sucrose and 0.8% agar. The specimens were kept in culture room in the following conditions: photoperiod 16-8 hours (light-dark), 25±1° C.

Fifteenth days old in vitrogerminatedseedlings of broccoli,Brassica oleracea var.italic, were removed from the culture medium. Leaves, stems and roots were excisedfor callus initiation;explants were cultured on agar solidified MS medium supplemented with various concentrations and combinations of plant growth regulatorsas shown below:

MS1: 0.5 BAP + 0.2 NAA mg L^{-1}

MS2: 1.0 IBA mg L^{-1}

MS3: 1.0 kin mg L^{-1}(Pavlovic etal, 2010)

MS4: 1.5 BAP+ 0.5 NAA mg L^{-1}

MS5: 2.0 TDZ+1.0 2,4-D mg L^{-1}

MS6: 1.5 BAP+1.0 IBA mg L^{-1}(Sharif Hossain et al., 2016)

MS7: 1.5 BAP+ 2.0 IBA mg L^{-1}

MS8: 0.5 TDZ mg L^{-1}

MS9: 1.5 TDZ mg L^{-1}

MS10: 2.0 TDZ mg L^{-1}

Specimens were maintainedin the culture room at 25∓2 ^{o}C, with 16/8 hours of light / darkness, with light intensity of 1000 lux.

Callus maintenance

The callus was maintained by sub-culturing 50mg of the callus on the best MS medium for callus growth depending on the response of each explantat an interval of four weeks.

Indirect somatic embryogenesis

Callus initiated from stem explants on MS1 mediumwas transferred to MS8 medium (MS + 0.5 mg L^{-1}TDZ) after one month as well as to MS medium supplemented with 0.4 mg L^{-1}TDZ. The implants were then kept in the growthroom. The regenerated shootswere transferred to MS medium freefrom growth regulators. When the number of branches for each shoot reached 3-5 with goodgrowth of leaves and roots, they were removed from the medium and

their roots were washed with distilled water.Regenerated plants were planted in peat-moss and kept in the growth room in the same previous conditions.A binocular dissecting microscope was used to investigate the different phases of somatic embryos.

Shoot proliferation and Rooting

For shoot regeneration solidified MS medium containing 4.0 mg L^{-1} BA + 0.5mg L^{-1} NAA as well as MS + 2.0mg L^{-1}TDZ + 0.5mg L^{-1}IAA medium were used. All implants were kept under the same conditions previously mentioned.The regenerated shoots were dissected and transported to 100 ml flasks containing 25 ml of MS medium free from growth regulators and others to MS medium supported with 1.0 and 2.0 mg L^{-1}IBA, and 2% of sucrose for rooting. All samples preserved with the same former conditions.

Acclimatization of plantlets

The plantletsof about 4-6 cm length with 5-9 leaves were transferred to pots containing mixture of peat- moss and soil in a ratio of 1:1. The pots were then covered with puncturedpolythene bags for 3-5 days in the growth room. After acclimatization, the polythene bags were removed and the plants allowed growingin natural conditions and they were watered regularly. Data on the ratio of survival plants was documented after 4weeks of transfer.

Results

Callus initiation

The results showed a high efficiency of the surface sterilization of broccoli seeds with a solution of commercial bleach 3 % NaOCl due to obtain sterilized seeds with efficiency reached 100 %. The sterilized seeds germinated after five days of cultivation on solid MS medium. Leaf, stem and root explants of these seedlingsshowed a clear response to callus induction in solid MS medium supplemented with different concentrations of auxins and cytokines.The stemexplants showed the ability to produce callus on MS medium supported with different levels of NAA and BAP.Themedium,MS1was superior to other media since the percentage of callusinitiation reached (98% p-value 0.00) and the time required for the development of callus 10 days (Table 1). The stem callus formed in this medium was friable and green yellowishin color (Fig.1, a).

The stem explants also showed the ability for callus initiation when cultured on MS8, MS9, and MS10 media, but a longer period of time was necessary to produce callus than on the

MS1medium.Leaf explants showed a weak response to callus induction,the callusbegan to form at the edge of the leaves (Fig.1,b) and covered the whole explant after a month of culture on the mediumMS9 (Fig.1,c).The leaf calluswas characterized by a hard texture and had a creamy yellowcolor. Concerning root explants, the results showed high response of these explants on MS medium supplemented with TDZ and the percentage of callus induction ranged from 80-100% (Table 1). The root callus was friable with bright green color (Fig 1,d).

Shoot regeneration

The results indicated high ability of callus derived fromstem explants of Brassica oleracea var.italicafor shooting in the differentiation medium MS supplemented with 4.0 mg L^{-1} BAP+0.5 mg L^{-1} NAA, wherethe percentage of shoot regeneration reached (88.5% p-value 0.00) (Table 2). Moreover, shooting percentage was 98.6 and (98.3% p-value 0.00) from root callus on MS9 and MS10 respectively(Table 2). Shoots began to appear from the callus two weeks after transferring the callus to regeneration medium (Fig.1, e), and grewgradually (Fig.1,f).

The average number of shoots formed reached 3-4 / piece of callus, and the length was 3-5 cm. At this stage, theywere moved tothe rooting medium(Fig.1,g).All regenerated shoots rooted easily in MS medium free fromgrowth regulators and MS containing 1.0, 2.0 mg L^{-1}IBA and 2% of sucrose (Fig.1,h).The acclimatization of regenerated broccoli plants was successful.

Somatic embryogenesis

The results showed the formation and development of embryos from stem and rootcallus after 30 days of culture on MS medium containing 0.5, 0.4 mg L^{-1} TDZ(Table2). The numberof somatic embryos was 20-25 / piece of callus containing different phases (globular, heart, torpedo, cotyledonary) which were examined using a binocular dissecting microscope.

Thesomatic embryos developed from meristematic cellsthat formed embryogenic clumps (Fig2.a), which were unique in their differentiation from the rest of the callus cells. Continuous division of these calli after 15 days led to the formation of globular stage (Fig.2,b). Generally, the other stages of somaticembryos: heart (Fig.2, c), torpedo (Fig.2,d) and coteledonary (Fig.2,e,f) were observed after subculturing. Finally, real shoots were produced (Fig.2 g, h,).Optimum root induction for theseshoots (98.8%p-value 0.00) (|Table 4) occurred on MS medium fortified with 1.0 and 2.0 mgL^{-1}IBA (Fig.2,j).Therefore,

acclimatization of the plants wastotally successful (Fig.2,k).All plants were transferred to the greenhouse conditions. These plants showed their ability to survive and endure the environmental conditions.

Discussion

The most significant step in plant tissue culture technique is surface sterilization of explant. In this paper, the sterilization process of seeds was 100% efficient as the seedswere not contaminated later, and produced healthy seedlings of high viability when grown on solidifiedMS medium.Other studies have found that industrial bleach (6 % sodium hypochlorite as active ingredient) and ethanol are very efficient sterilizing agents for establishing aseptic seedlings (Kim and Botella,2002; Farooq etal., 2019). Antibacterial properties of hypochlorite are well known. The concentration of hypochlorite used (NaOCl) when diluted in water produces hypochlorous acid (HOCl) (Nakagarwara et al., 1998).

The callus induction is considered the first stepin many tissue culture approaches such as the establishing of cell suspension cultures (Ngara et al., 2008), indirect somatic embryogenesis (Rahman et al., 2006) and other applications. In our study, clear differences between the responsesof various broccoli explants to callus initiation and this ismay be due to various factors such as tissue physiological status and endogenous hormone levels, which was also proved by Mustafa et al., (2020). Furthermore, several parameters such as the media, growth regulators, culture conditions, and type of explant have an impact on in vitro cultures (Janowicz, et al., 2012). Furthermore, in callus induction, explant selection is critical, and the explant's response is highly reliant on its genotype and physiological state. As a result, different types of explants for any given species behave differently, resulting in variable amounts of embryogenic calli induction (Dhiya-Dalila et al.,2013)

The concentrations of growth regulators (both cytokinin and auxin) are essential for callus yield and plant regeneration. Theoretically, equal amount of auxin and cytokinin promote callus induction but, in fact, this varies greatly because to differences in endogenous phytohormone levels in individual plants (Afshari et al.,2011; Kumar et al.,2014). The reason for the performance of plant explant for callus induction may be as a result of the compatibility between their internal content of plant hormones and growth regulators added to the medium (Azis et al., 2015).

The results revealed that concentration of growth regulators in the differentiation medium (MS + 4.0 mg L^{-1} BAP + 0.5mg L^{-1} NAA) is very suitable for shoot regeneration from stem callus. BAPseems to be effective in enhancing shoot multiplication and triggering shoot

elongation (Asharf et al., 2014). Asharf et al.(2014) also stated that BAP also promotes differentiation of cells into shoot initials followed by the formation of shoots.In vitro regeneration in the genus Brassica is highly genotype specific and huge differences have been recounted in the regeneration potential of diverse genotypes(Farooq et al., 2019). Ravanfar and his colleagues (2014)observed that a combination of 0.1 mg/l TDZ with 0.1 mg/l NAA was very effective for shoot regeneration from cotyledonary explants of broccoli. Interestingly, spontaneous formation of shoots in the same media for callus induction in Arnebia benthamiiplant was due to the genotype of the plant variety used in addition to the levels of growth hormones within the callus tissue (Parray et al., 2018).

Somatic embryogenesis is the mechanism by which a somatic cell or a group of somatic cells develop into an embryo capable of growing into a complete plant (Romero, 2021). It is the most widely used and effective method for clonal plant propagation (Tomiczaket al., 2019).It is usually preferred over the methods of reproduction (Al Shamari et al. 2018).In this study the use of TDZ was very effective in inducing indirect somatic embryogenesis from stem and root calli of broccoli. Thidiazuron received a lot of attention in recent decades because of its importance in in vitro culture with Auxin and cytokinin-like effects in different plant species e.g. Rhododendron sichotense Pojark and strawberry Fragaria vesca(Ghosh, et al., 2018;Zaytsevaet al., 2020; Chung and Ouyang, 2021; Taha et al., 2021).The action mechanism of TDZ is that it aids in the accumulation and/or synthesis of endogenous growth hormones (Guo et al, 2011). In fact, TDZ induces somatic embryogenesis in a variety of plant species, including the blume orchid (Mose et al., 2017) and olive (Narváez et al., 2019).Guoet al. (2005) reported that the combination of TDZ and NAA produced more shoots than BAP alone on cotyledon and leaf segmentof mustard (Brassica juncea var. tsatsai).Shoja and Shishavan (2021) indicated that TDZ was the best type of growth regulator compared to BA and Kin for in vitro cultures of Hyssopus officinalis

The ease of the regenerated shoots on MS medium may be due to the presence of enough endogenous auxins in these shoots that enabled them to form roots(Handayani, 2014)

Conclusions

An efficient approach for inducing shoot regeneration in broccoli Brassica oleracea var.italica was used via organogenesis and indirect somatic embryogenesis. This work proved that TDZ was effective in these approaches.This plant is not cultivated in our country; the plant tissue culture technique could be a substitute for this purpose.

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Table1. Assessment of different MS media for callus induction from various explants of Brassica oleracea var.italica.

Media	**Explant**	Period for callus induction (days)	Percentage of Callusinitiation* **(%)**
MS1	Stem	10	98.00 ±2.28035
	Leaf	20	40.33 ±1.63299
	Root	23	9.167 ± 0.98319
MS2	Stem	20	38.833 ±1.16905
	Leaf	25	31.33 ±1.16905
	Root	-	-
MS3	Stem	22	58.833 ±1.60208
	Leaf	-	-
MS4	Stem	24	58.833 ±1.16905
	Leaf	-	-
MS5	Stem	22	20.33 ±1.63299

MS6	Stem	10	79.00 ±1.26491
	Leaf	-	-
	root	-	-
MS7	Stem	15	69.00 ± 1.26491
	Leaf	-	-
MS8	Stem	12	99.00 ±1.26491
	Leaf	20	19.00 ±1.26491
	root	15	78.33 ±1.63299
MS9	Stem	15	99.00 ±1.26491
	Leaf	20	69.33 ±.81650
	root	15	99.33 ±.81650
MS10	Stem	15	74.00 ±1.26491
	root	20	99.00 ±1.26491
p-value			0.00

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*The presented data are mean± standard deviation

Table2. Shoot regeneration from callus of Brassica oleracea var.italic. ondifferent combinations of MS medium.

Media	**Shoot regeneration* (%)**		
	Leaf callus	**Stem callus**	**root callus**
MS+ 4.0 mg L^{-1}BAP+0.5 mg L^{-1} NAA	39.0000 ±1.26491	88.5000 ±1.76068	0.0000 ±0.00000
MS+ 2.0 mg L^{-1}TDZ+ 0.5 mg L^{-1} IAA	0.0000 ±0.00000	0.0000 ±0.00000	0.0000 ±0.00000
MS+ 0.5 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	74.0000 ±1.26491	99.0000±1.26491	0.0000 ±0.00000
MS+ 1.5 mg L^{-1} TDZ	18.6667±1.50555	29.0000±1.26491	98.6667±1.75119
MS+ 2.0 mg L^{-1}TDZ	9.1667 ±0.98319	19.0000±1.26491	98.3333±1.63299

p-value	0.00	0.00	0.00

*The presented data are mean± standard deviation

Table 3.The number of somatic embryos produced from stem and root callus of B. olearcea var. italica in MS medium containing TDZ at different stages.

Media	source of callus	Numbers of embryos / stage*				p-value
		Globular	Heart	Torpedo	Cotelydonary	
MS+ 0.5mgL^{-1}TDZ	Stem	28.333 ±1.1547	25.33 ±1.1547	20.67 ±0.57735	19.00 ±0.00	0.00
	Root	34.67 ±2.309	30.33 ±1.1547	29.33 ±1.1547	28.00 ±1.7332	0.00
MS+ 0.4mgL^{-1}TDZ	Stem	31.667 ±0.5774	31±0.00	22.33± 1.5275	20.67 ±0.57735	0.00
	Root	36.00 ±2.00	33.33 ± 2.5166	31.33 ±1.5275	30.33 ±1.5275	0.00
MS0 (control)	Stem	0±0.00	0±0.00	0±0.00	0±0.00	0.00
	Root	0±0.00	0±0.00	0±0.00	0±0.00	0.00

*The presented data are mean± standard deviation

Table 4. Rooting of shoots regenerated from callus and somatic embryos ofB. olearcea var. italica in MS medium.

Media	**Sucrose (%)**	**Rooting induction (day)**	**Rooting* (%)**
MS0	3	15	72.4000 ± 2.07364
MS+ 1.0 IBA	2	10	98.8000 ±1.30384
MS+ 2.0 IBA	2	7	98.8000 ±1.64317
p-value			0.00

*The presented data are mean± standard deviation

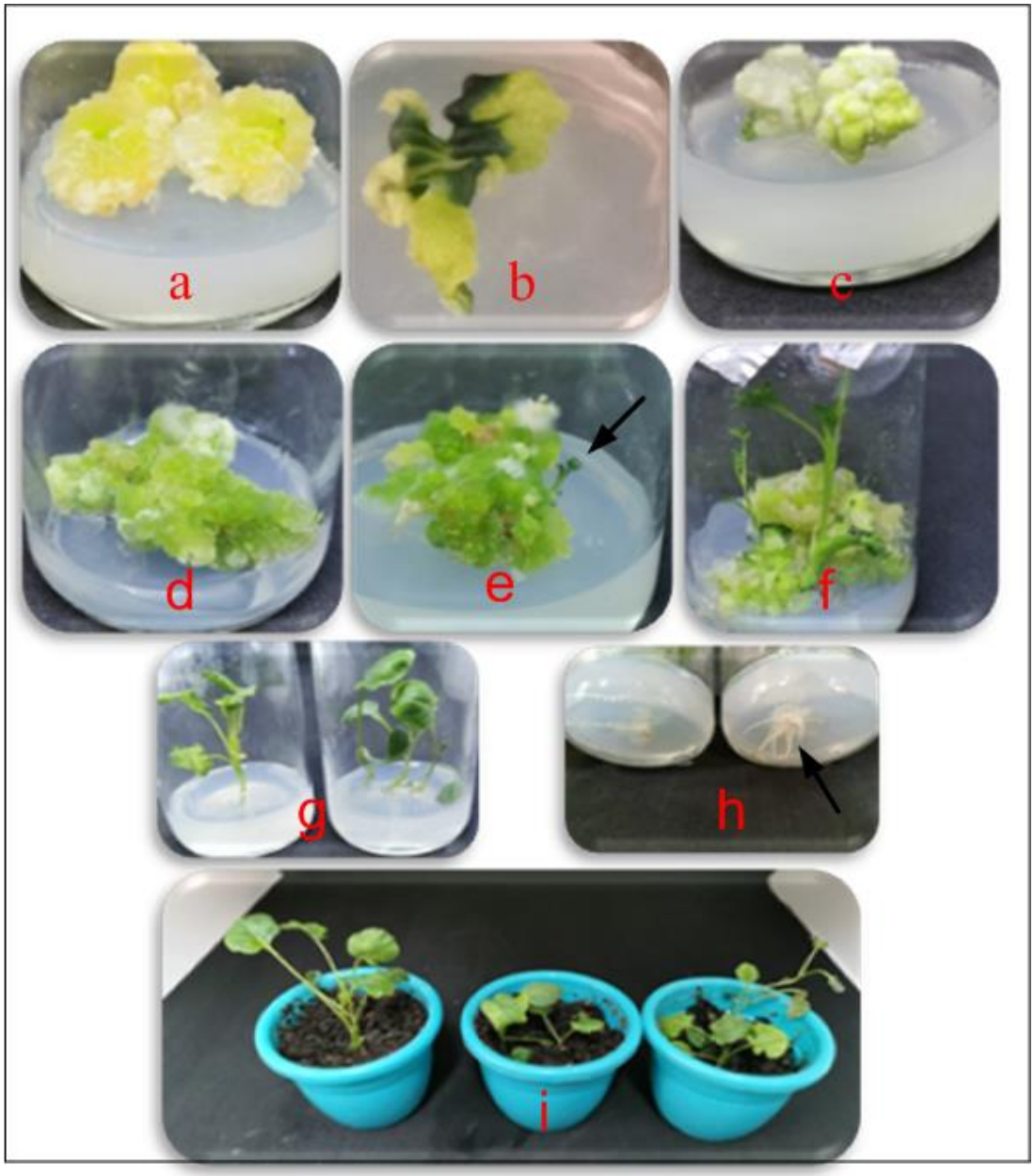

Fig. 1. Callus induction and plant regeneration of Brassica oleracea var.italica via organogenesis in MS medium with different combination of growth hormones: (a) Stem callus in MS +0.5 BAP + 0.2 NAA mg L^{-1}(b)Beginning of callus induction from leaf explant in MS +1.5 TDZ mg L^{-1}(c) Leaf explant in after a month of culture in MS +1.5 TDZ mg L^{-1} (d)Callus of root in MS +1.5 TDZ mg L^{-1}(e) shoot regeneration from root callus in MS+ 4.0 mg L^{-1} BAP+0.5 mg L^{-1} NAA(arrows)(f)Development of shoots in regenerated from root callus in MS+ 4.0 mg L^{-1} BAP+0.5 mg L^{-1} NAA (g) Regenerated shoots in rooting medium (MS containing 1.0 mg L^{-1} IBA)(h)Rotting of shoots (arrows)(i) Acclimatization of regenerated plants.

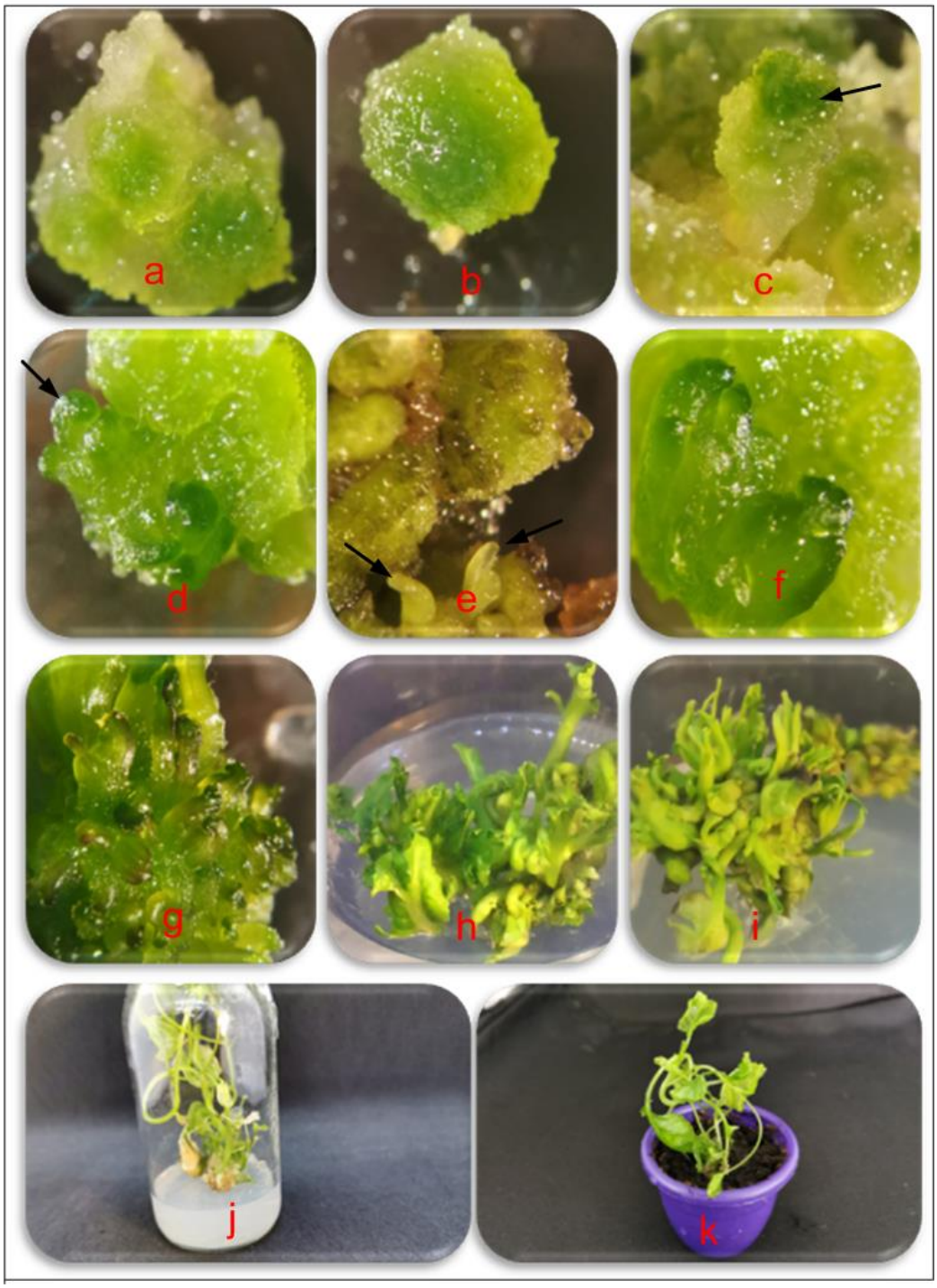

Fig. 2. Somatic embryogenesis of callus derived from root explants of Brassicaoleracea var.italicain MS+ 0.4mgL^{-1}TDZ: (a) Embryogenic clumps after 30 days of culture (b) Globular stageof somatic embryos (c) Heart stage of somatic embryos (arrows)(d) Torpedo stage of somatic embryos (arrows)(e, f)Coteledonary stageof somatic embryos (g, h,i) Development of somatic embryos into shoots. (j)Rooting of shoots in MSmedium containing 1.0 mgL^{-1} IBA(j) Acclimatization of plants.