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# Optimization of kanamycin dose for *in vitro Camelina sativa* transformation

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**Abstract:** *Camelina sativa* is an underutilized oilseed crop that can be grown under different climate conditions. As its requirements for growth are relatively low with a short life cycle, it can be utilized in marginal lands for crop rotations. Camelina shows great promise as a source of food, feed, chemicals, and biofuel. Enabling the genetic transformation of *C. sativa* would facilitate the fast incorporation of new characteristics into this growing crop. Moreover, genetic and metabolic engineering can be applied to decrease unwanted secondary metabolites as well as boost the beneficial products. Kanamycin is one of the most used antibiotics in plant transformation. Here, the effects of kanamycin on the seeds of Camelina were analyzed by observing different parameters such as germination, seedlings, shoot, and root growth as well as its fresh and dry weight. Prevalent effects of kanamycin were shortening of root and shoot length, thinning of shoots, and discoloration. Also, true leaves could not grow in the presence of the antibiotic. Based on these results using 100mg/L kanamycin as an additive to the growth media in tissue culture would allow the selection of transformant plants and allow them to grow as transgenic plants for desired purposes.

**Keywords:** Camelina, genetic transformation, kanamycin, nptII gene



## 1. Introduction

*Camelina sativa* (Camelina) is an oilseed plant classified under the Brassicaceae family that has attracted significant appreciation in recent years (Murphy 2016).

It is an annual plant with a very short life cycle of 85-100 days with the height at maturity ranging from 60 to 110 cm. It is predominantly a self-pollinating plant. The plant produces little yellow flowers arranged in a raceme formation, each consisting of four petals. Stems can be either smooth or hairy and it possesses branches that become woody as they mature. It has pear-shaped fruits around 5 mm in diameter. C. sativa seeds are small, weighing only 0.8 to 1.8 grams per 1000 seeds (Mondor and Hernández-Álvarezi 2022). Camelina is an optimal crop for less fertile lands and regions with little rainfall, as it demands very little inputs. It is a resilient plant that has strong adaptability to many soil types and thrives in cold semi-arid regions and also exhibits a high tolerance for arid conditions (Rostami Ahmadvandi and Faghihi 2021; Urbaniak et al. 2008). It has nutritious seeds that have a high oil content ranging from 30% to 49% and a protein content ranging from 24% to 31% which is similar to soybean (Mondor and Hernández-Álvarezi 2022; Zubr 1997). Its oil has a significant amount of healthy polyunsaturated fatty acids (PUFAs), specifically linoleate (18:2) and linolenate (18:3) (Bansal and Durrett 2016). The efforts in the breeding programs of Camelina are focused on the increment of production, improving the oil content and its quality, and decreasing the glucosinolate (anti-nutritive) composition of the seeds (Ghidoli et al. 2023).

The depletion of inexpensive fossil fuel reserves and growing worries regarding climate change have heightened the necessity to acquire fuel and chemical products from renewable sources. Historically, Camelina has been utilized for both human and animal consumption and various non-food purposes, including the manufacturing of soap, cosmetics, lamp oil, and non-toxic paints (Zubr 1997). It is a superior crop for bio-diesel production due to its rapid growth and great yield potential of 1500-3000 kg ha−1 year−1 (Sainger et al. 2017). C. sativa is an ancient crop plant origin of which is assumed to be in the region between Russia and Ukraine whereas its cultivation dates back to

4000 BCE (Berti et al. 2016; Ghamkhar et al. 2010). It is a hexaploid plant with 2n=40 chromosomes and a genome of 750 Mbp (Hutcheon et al. 2010).

Agrobacterium tumefaciens may efficiently introduce a DNA segment of interest into C. sativa via floral dip approach or in vitro leaf explant cultures, resulting in the production of transgenic seeds within a very short timeframe of 4-6 weeks. Transgenic seeds can be distinguished using selectable markers such as the red fluorescent protein (DsRed), mCherry fluorescent protein, or antibiotic resistance (Sitther et al. 2018; Lu and Kang 2008). In this study, different doses of kanamycin were applied in *in vitro* conditions to *C. sativa* seeds to elucidate its effect on germination and growth. Kanamycin is one of the most common antibiotic markers in plasmids used for genetic transformation. Thus, adjusting its dose of use is a prerequisite for generating transgenic *Camelina* plants through *in vitro* method.

## 2. Materials and Method

To study the effect of kanamycin in germination and growth, the Turkish cultivar Arslanbey of *C. sativa* was used.

### *2.1. Sterilization and culture*

Surface sterilization was accomplished by treating the seeds with 70% commercial bleach (<5% w/v NaOCl) for 10 minutes then washing the seeds three times for 10 minutes with distilled sterile water. Ten seeds were placed on MS (Murashige and Skoog 1962) medium with different concentrations of kanamycin.

### *2.2. Preparation of media*

To prepare MS medium, 0.44 % MS mineral salts, and vitamins were supplemented with 3% sucrose and 0.65% agar (Aasim et al. 2011). The pH of the solution was adjusted to 5.6 with 1M NaOH and 1M HCl. Following autoclave for 21 minutes at 121$^0$C, the media was left to cool down for the addition of the antibiotic and then poured to plastic petri dishes for polymerization.

### *2.3. Antibiotic doses*

Four doses of kanamycin MS25 (25 mg/L), MS50 (50 mg/L), MS75 (75 mg/L), and MS100 (100 mg/L) using filter sterilized (MF-Millipore™ Membrane Filter, 0.22 µm) kanamycin stock solution (50 mg/mL), as well as one MS medium without kanamycin (as negative control) was prepared.

### *2.4. Experimental design and analysis*

All experiments were conducted in triplicate. Cultures were placed in the growth room under 16h day – 8h night photoperiod at 24$^0$C. Seeds, their germination, and growth were monitored for 30 days, and the readings were recorded at the end of the experiment. Germination and seedling rates were also recorded. Shoot length, root length, and total length were measured using a ruler (Fig. 1). Fresh and dry weight of each seedling was measured. For dry weight measurement, seedlings were placed into an oven at 65$^0$C for 48 hours. One-way ANOVA analysis was performed using the Tukey test using Minitab 20.4 software.

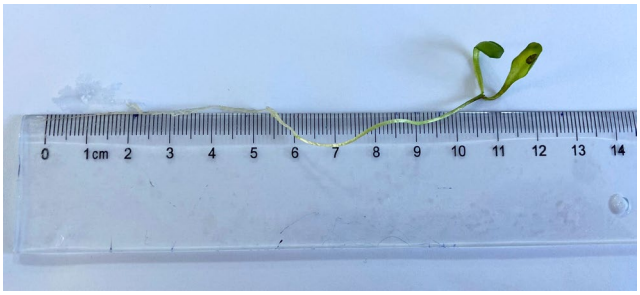


**Fig. 1** Measurement of seedlings with a ruler

## 3. Results

The negative control, which is the MS medium without kanamycin, was used to examine seedlings one month after sowing in vitro (Fig. 2). There was considerable growth where roots and shoots were intertwined. Plants were healthy, with rooting system ready for acclimatization to the soil.

**Fig. 2** Growth of *C. sativa* seeds on MS medium

The effects of kanamycin on seedlings were obvious even at low doses (25 mg/L). Roots were much shorter, likewise, shoot growth was retarded. The decrease in the length of shoots and roots was more emphasized at higher doses. No leaves other than cotyledonary leaves were present in the presence of kanamycin. True leaves were unable to grow within a one-month period. Discoloration was noticeably high at higher doses. The thickness of the shoots was also observed to be significantly reduced. Despite these effects, there was little reduction in germination and seedling even at higher doses (Fig. 3).

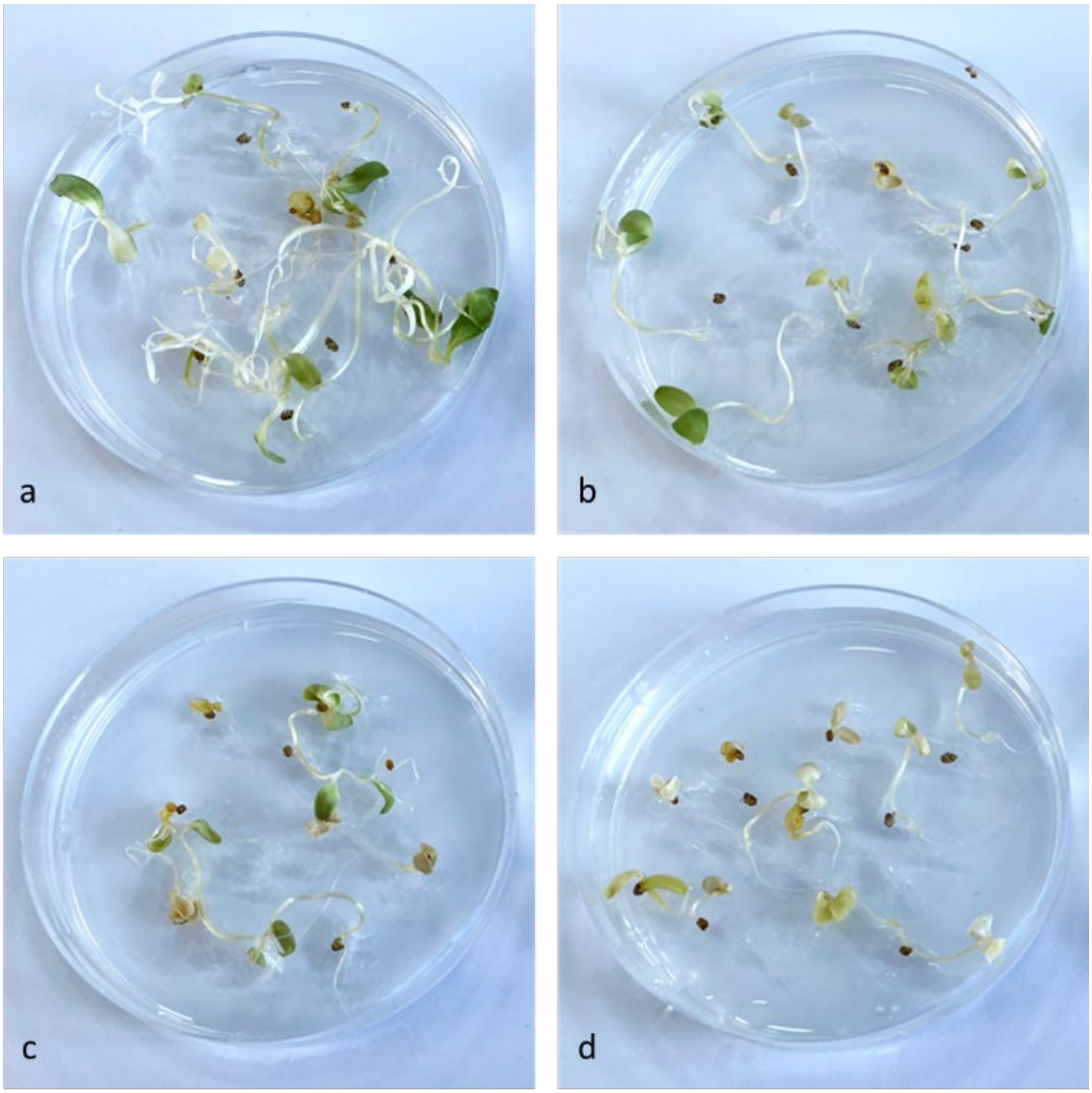


**Fig. 3** Effect of different doses of kanamycin on *C. sativa* seeds; a) 25 mg/L, b) 50 mg/L, c) 75 mg/L, d) 100 mg/L

**Table 1** *C. sativa* growth parameters on different kanamycin concentrations (30 days after sowing)

| | Root length (cm) | Shoot length (cm) | Total length (cm) |
|---|---|---|---|
| **MS** | 4.8 ± 1.605 a | 9.1 ± 1.636 a | 13.9 ± 2.77 a |
| **MS25** | 2.2 ± 0.975 b | 5.5 ± 2.37 b | 7.7 ± 1.987 b |
| **MS50** | 2.3 ± 0.57 b | 3.2 ± 0.837 bc | 5.5 ± 1.173 bc |
| **MS75** | 1.9 ± 1.025 b | 2.5 ± 1.173 c | 4.4 ± 2.043 bc |
| **MS100** | 1.5 ± 0.935 b | 2.5 ± 0.354 c | 4 ± 1.173 c |

* Letters following means in the same column indicate that the means are statistically significantly different ($p<0.05$)

**Table 2** *C. sativa* germination seedling and plant weight on different kanamycin concentration (30 days after sowing)

| | Germination (%) | Seedling (%) | Fresh weight (g) | Dry weight (g) |
|---|---|---|---|---|
| **MS** | 100 | 100 | 0.3134 ± 0.115 a | 0.01946 ± 0.015 a |
| **MS25** | 100 | 90 | 0.0584 ± 0.025 b | 0.00126 ± 0.0002 b |
| **MS50** | 100 | 100 | 0.0324 ± 0.019 b | 0.00264 ± 0.0003 b |
| **MS75** | 90 | 70 | 0.0354 ± 0.017 b | 0.00468 ± 0.0019 b |
| **MS100** | 100 | 90 | 0.0238 ± 0.0093 b | 0.00235 ± 0.0008 b |

* Letters following means in the same column indicate that the means are statistically significantly different ($p<0.05$)

The longest roots were obtained, as expected, in seedlings grown on MS medium with a mean value of 4.8 cm. The shortest root length was noticed at MS100 with a mean value of 1.5 cm. Root length on different kanamycin concentrations was statistically significant regarding the presence or absence of kanamycin. Shoot length ranged from 9.1 cm on MS mediumto 2.5 cm on MS100 and there was a significant difference of shoot length between doses. The mean total length of the seedlings was 13.9 cm on MS medium, 7.7 cm on MS25, 5.5 cm on MS50, 4.4 cm on MS75, and 4 cm on MS100 and the difference was statistically significant (Table 1).

The antibiotic did not affect germination, and nearly all seeds had germinated at all conditions. The seedling stage

(growth of shoot) was slightly impaired in higher kanamycin doses decreasing to 70% and 90% at 75 mg/L and 100 mg/L respectively. Fresh weight was 0.31 g per seedling on MS medium decreasing gradually with increment of concentration of kanamycin down to 0.024 g, showing more than ten-fold decrease at 100 mg/L. Differences in fresh weight between doses of kanamycin were statistically significant. Similarly, dry weight decreased drastically from 0.019 g on MS medium to 0,0024 on 100 mg/L. Likewise, differences in dry weight between doses of kanamycin were statistically significant (Table 2).

## 4. Discussion

There is an ongoing demand for employing model plants that are suitable for genetic and metabolic engineering, that possess simple and high-capacity transformation systems, a fully sequenced genome, short life span, high yield, and the capability to analyze the produced plants in field experiments (Malik et al. 2018).

Generally, Arabidopsis thaliana is used in transgenic studies, but Camelina sativa is emerging as a new promising plant as a model for oilseed crops for seed oil production where metabolic engineering can be achieved easily (Bansal and Durrett 2016). Its compliant nature for genetic transformation has created attention for researchers to apply genome editing systems such as CRISPR/Cas9.

Besides the floral dip method, which has an efficiency of around 1%, tissue culture protocol can be used for the transformation of C. sativa (Liu et al. 2012). Even though tissue culture is more difficult to accomplish, it is advantageous in its efficiency, getting more seedlings in small areas with almost all surviving plants being transgenic positive. One of the most important aspects of tissue culture is adjusting the dose of selective antibiotic, such that it will not let the untransformed plants grow, but also would not hamper the growth of the transformed plants (Bakhsh et al. 2015; Anayol et al. 2016; Ahmed et al. 2017). Kanamycin is one of the most robust and commonly used antibiotics in gene transformation efforts. Non-transformant plants are normally susceptible to kanamycin, whereas the transformed plants that have the nptII gene (neomycin phosphotransferase II enzyme) incorporated in their genome will survive and grow under certain kanamycin concentrations. There are 133 genetic transformation events globally that incorporated the nptII gene along with the desired traits for commercial use (ISAAA GM Approval Database 2023).

In Camelina, kanamycin causes the shortening of shoots and roots. Moreover, true leaves did not emerge even at lower kanamycin concentrations. Discoloration of leaves and stems accompanied by the death of tissues at high kanamycin concentrations was noticed (Fig. 3). At 100 mg /L dose no green leaves remained after a one-month period. In their publication, Sitther et al. (2018) used shoot apical meristems of C. sativa for transformation with EGFP and nptII genes. They used 40 mg/L kanamycin to select the transformant plants, however, some green parts remained in untransformed plants. Ontiveros-Cisneros et al. (2022) used up to 150 mg/L kanamycin on wild-type Camelina seeds and still, green leaves were visible and could not distinguish between wild-type and transformant seeds. Probably the seeds of the wild type of C. sativa used in their study have higher antibiotic resistance as it is the only publication where kanamycin could not be used successfully for transformant selection.

Kanamycin did not impede the germination of Camelina seeds, while it had minimal effect on their transition to the seedling stage. Similar effects were recorded by Ontiveros-Cisneros et al. (2022) where all the seeds germinated even at higher doses (150 mg/L) of kanamycin. The effect on root length, fresh weight, and dry weight was statistically significant, however, all the kanamycin doses (except MS0) were grouped in the same group according to Tukey's test. In shoot length and total length addition of kanamycin at different concentrations resulted in statistically different results. In their study Sitther et al. (2019) also found a diminishing of shoot and root length however, plant tissues were undergone necrosis even at 40 mg/L of kanamycin. Their cultivars (PI650159 and PI650161) seem to be more susceptible to the antibiotic. Using shoot apical meristems as explants may require lower doses of kanamycin. On the other hand, Zakharchenko et al. (2013) used 50 mg/L of kanamycin for the selection of transgenic seeds but there is no information on the condition of non-transformant plants.

## 5. Conclusion

Based on these findings, 100 mg/L concentration is considered to be suitable for selecting transformed Arsalanbey cultivar seeds and tissues from non-transformed ones, as there were no green patches on leaves and ultimately no photosynthesis. It is important to optimize the concentration of the antibiotics for each cultivar before starting the selection procedure. This study presents a framework for the genetic transformation of C. sativa via tissue culture. Diversifying methods of transformation will open the opportunity to use them for study-specific purposes.

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